Regulation and function of DeltaNp73 isoforms after DNA damage

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THESIS

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INTRODUCTION

I- Control of DNA damage

II- p73 : member of p53 transcription factors family

RESULTS

Regulation and function of ΔNp73 isoforms after DNA damage.

In our search for the underlying causes of cancer, TP53 is the most intensively studied gene. p53 plays a central role for balancing the antagonistic processes of proliferation and apoptosis. As a sequence-specific transcription factor, p53 regulates the expression of genes involved in cell cycle arrest and apoptosis in response to genotoxic damage or cellular stress. Failure of p53 function consequently leads to uncontrolled cell growth, a defining feature of cancer cells. Given the importance of p53 as a tumor suppressor, it is therefore no wonder that p53 is the most frequent site of genetic alterations found in human cancers. The recent discovery of two TP53-related genes, TP73 and TP63 with striking sequence homology, was therefore a big surprise, raising the possibility that other tumor suppressors exist which share the power of p53 in preventing cancer formation.

The three members of the p53 family share significant homology both at the genomic and at the protein level. The highest level of identity is reached in the DBD (DNA-Binding Domain), suggesting that they can bind to the same DNA sequence and transactivate the same promoters. In fact, p73 and p63 are able to activate some p53 targets and to induce apoptosis, but they appear more and more different from their relative. The study of the respective knock-out mice gives a good illustration of these differences : while p53-null mice develop normally but present spontaneous tumors, the p73 and p63-null mice present severe developmental troubles but no spontaneous tumors, indicating that they may have more complex functions.

Conversely to p53, p73 and p63 contain additional C-terminal extensions. In both proteins, these extensions show alternative splicing, which results in at least six C-terminal variants for p73 and three for p63. These isoforms have different transcription and biological properties, and their expression patterns change among normal tissues. Moreover, the α variants of p73 and p63 have close to their C terminus a SAM (Sterile Alpha Motif) domain, which is thought to be responsible for regulating p53-like functions, and is implicated in various human syndromes where p63 is mutated. In addition to the C-terminal variants aminoterminous truncated variants of p73 and p63 exist : ΔNp73 and ΔNp63. These N-terminally truncated isoforms lack the transactivation domain (TA), which is coded by the first 3 exons, and derive from the use of an alternative promoter (P2)

located in intron 3 and an additional exon (exon 3'). While TAp73 isoforms work as transcription factors and can induce irreversible cell cycle arrest and apoptosis like p53, the ΔNp73 isoforms that lack the transactivation domain are incapable of directly inducing gene expression and do not induce growth arrest or cell death. However, the ΔNp73 forms have a very important regulatory role, since they exert a dominant negative effect on p53 and TAp73 by blocking their transactivation activity, and hence their ability to induce apoptosis. The relative levels of expression of the ΔNp73 isoforms can therefore determine the function of both TAp73 and p53. It is most interesting that the ΔNp73 promoter (P2) contains a very efficient p53/p73 responsive element and consequently, p53 and TAp73 efficiently induce ΔNp73 expression. Moreover, upon strong DNA damage, induced by UV irradiation or drug treatment, ΔNp73 is rapidly degraded, releasing the block exerted on p53 and TAp73 and thus allowing cell cycle arrest and apoptosis to proceed. Hence, ΔNp73 is part of a dominant negative feedback loop that regulates the function of both p53 and TAp73 and this regulation can be overcome in case of strong DNA damage.

Regolazione e funzione delle isoforme ΔNp73 dopo danno al DNA.

Le cellule di un organismo subiscono ogni giorno stress dovuti all'ambiente (raggi UV, agenti chimici, metalli pesanti) che possono causare lesioni al patrimonio genetico o un disequilibrio dello stato RedOx. Numerosi sistemi cellulari permettono prima di tutto di identificare il danno, ed in seguito d'indurre eventualmente la riparazione del DNA o la morte della cellula se il danno subito è irreversibile. Una cellula tumorale è il risultato dell'accumulo dei fallimenti dei sistemi di controllo intra- ed extra-cellulari e della morte programmata. Identificata nel 1979, la proteina p53 è un fattore di trascrizione mutato in 50% dei tumori. Ha un ruolo centrale nella regolazione della proliferazione cellulare, della riparazione del DNA e dell'apoptosi dopo insulto, genotossico o meno. Stranamente, p53 sembrava avere da sola questo ruolo preponderante che l'è valso la denominazione di "guardiano del genoma", e, negli ultimi 20 anni, tutti i tentativi per caratterizzare eventuali omologhi sono falliti. Nel 1997, p73, un omologo di p53, fu identificata nella banda p36 del cromosoma 1, una regione la cui delezione è spesso associata a numerosi neuroblastomi. La caratterizzazione di p73 fu accolta con entusiasmo e la sua grande omologia con p53 sembrava poter spiegare il 50% dei tumori che presentano p53 non mutata. L'anno successivo, un secondo omologo fu identificato e caratterizzato : p63. I tre membri della famiglia di p53 presentano una grande omologia, in particolare nel dominio di legame al DNA : p73 e p63 sono in effetti capaci di attivare l'espressione di numerosi geni bersagli di p53 e d'indurre l'apoptosi o di bloccare il ciclo cellulare.

Tuttavia, sei anni dopo la loro scoperta, p73 e p63 sembrano sempre più diversi dal loro "parente" p53. La generazione e lo studio di topi transgenici mancanti dei diversi membri della famiglia illustrano queste differenze : se i topi mancanti di p53 raggiungono senza problemi l'età adulta e sviluppano tumori spontanei, i topi mancanti di p73 o p63 presentano gravi disturbi nello sviluppo embrionale, indicando un loro ruolo principale nel differenziamento cellulare. Se p73 e p63 presentano una struttura globale paragonabile a p53 (un dominio di transattivazione, un dominio di legame al DNA e un dominio di oligomerizzazione, implicato nella tetramerizzazione della proteina indispensabile alla sua attività trascrizionale), p73 e p63 possiedono in effetti un prolungamento del dominio C-terminale, assente nella sequenza di p53, e che sembra coinvolto nelle loro funzioni. La maturazione dei trascritti di p73 e p63 genera differenti varianti di splicing (6 per p73 e almeno 3 per p63) nella porzione C-terminale le cui funzioni

trascrizionale ed il loro quadro di espressione sono differenti. Inoltre, le forme intere delle due proteine presentano un dominio SAM (Sterile Alpha Motif) comune a numerose proteine implicate nel differenziamento. Questo dominio è spesso mutato in alcune sindrome umane che coinvolgono p63, il che lascia presupporre un suo ruolo importante nella regolazione dell'attività di p63 e p73. In più, sono state descritte nel topo forme troncate nell'estremità N-terminale : queste forme sono chiamate ΔN e non possiedono il dominio di transattivazione, al contrario delle forme lunghe (TA). Dunque, ΔNp63 e ΔNp73 agiscono nel topo come dominanti negativi delle funzioni pro-apoptotiche di TAp63 e TAp73. In seguito, è stato stabilito che ΔNp63 e ΔNp73 sono trascritti da un secondo promotore, localizzato nel terzo introne della forma lunga.

In questo lavoro, è descritta per la prima volta la caratterizzazione della forma ΔNp73 umana, la regolazione della sua espressione e della sua attività. Come ΔNp73 murina, la forma umana inibisce le funzioni pro-apoptotiche di TAp73 e p53, attraverso l'interazione proteina-proteina o la competizione per i siti di legame sui promotori bersagli. Inoltre, la presenza di un elemento di risposta a p53 situato nel promotore di ΔNp73 indica l'esistenza di un feedback negativo che si aggiunge a quello già descritto per MDM2 e p53. Poiché ΔNp73 agisce come un oncogene, sembra che il rapporto ΔN/TA o ΔN/p53 sia fondamentale per l'equilibrio cellulare e che la sua deregolazione possa essere implicata nella formazione tumorale. Questo rapporto può essere controllato a livello trascrizionale o post-traduzionale della proteina ΔNp73. Lo studio del promotore di ΔNp73 a messo in evidenza numerosi elementi di risposta per differenti fattori di trascrizione. Poiché lavori recenti hanno associato l'aumento di ΔNp73 a diversi tipi di neuroblastomi, ci siamo particolarmente interessati a N-Myc, un fattore di trascrizione ugualmente amplificato in certi neuroblastomi. Tuttavia, non siamo stati in grado di mettere in evidenza un'attivazione trascrizionale diretta su ΔNp73 da parte di N-Myc, ma altri elementi di risposta rimangono ancora da caratterizzare, in particolare NFκB e p300, entrambi implicati nella regolazione dell'apoptosi. Inoltre, lo studio delle modificazioni post-traduzionali di ΔNp73 ha messo in evidenza una rapida degradazione della proteina in seguito a danno al DNA indotto da raggi ultravioletti o trattamento con droghe, liberando quindi p53 e TAp73 dalla sua inibizione e permettendo l'apoptosi o l'arresto del ciclo cellulare. In più, il nostro studio ha messo in evidenza una emi-vita molto breve della forma ΔN rispetto alle forme che contengono il dominio di transattivazione. Dunque, il rapporto ΔN/forme lunghe potrebbe ugualmente dipendere da una fine regolazione della degradazione delle due proteine.

Régulation et fonction des isoformes ΔNp73 après dommage à l'ADN.

Les cellules d'un organisme subissent chaque jour des stress dus à l'environnement (rayons UV, agents chimiques, métaux lourds) pouvant conduire à des lésions du patrimoine génétique ou à un déséquilibre de l'état RedOx. De nombreux systèmes cellulaires permettent tout d'abord d'identifier le dommage puis d'induire éventuellement la réparation de l'ADN ou la mort de la cellule si le dommage subi est irréversible. Une cellule cancéreuse est le résultat d'échecs cumulés des systèmes de contrôle intra et extra-cellulaires et de mort programmée. Identifiée en 1979, la protéine p53 est un facteur de transcription muté dans 50% des cancers. Elle joue un rôle central dans la régulation de la prolifération cellulaire, de la réparation de l'ADN et de l'apoptose après stress, génotoxique ou non. Etonnamment, p53 semblait jouer seule ce rôle prépondérant qui lui a valu la dénomination de "gardienne du génome", et, pendant 20 ans, toutes tentatives pour caractériser d'éventuels homologues sont restées vaines. En 1997, p73, un homologue de p53, fut identifiée dans la bande p36 du chromosome 1, une région dont la délétion est souvent associée à de nombreux neuroblastomes. La caractérisation de p73 fut accueillie avec enthousiasme et sa grande homologie avec p53 semblait pouvoir expliquer les 50% de cancers présentant une p53 non mutée. L'année suivante, un deuxième homologue fut identifié et caractérisé: p63. Les trois membres de la famille p53 présentent une grande homologie, notamment dans le domaine de liaison à l'ADN : p73 et p63 sont en effet capables d'activer l'expression de nombreux gènes cibles de p53 et d'induire l'apoptose ou de bloquer le cycle cellulaire.

Toutefois, 6 ans après leur découverte, p73 et p63 semblent de plus en plus différents de leur "parente" p53. La génération et l'étude de souris déficientes pour les membres de la famille illustrent ces différences : si les souris manquant p53 atteignent normalement l'âge adulte et développent spontanément des tumeurs, les souris manquant p73 ou p63 présentent de graves troubles du développement embryonnaire, indiquant un rôle majeur dans la différenciation cellulaire. Si p73 et p63 présentent une structure globale comparable à p53 (un domaine de transactivation, un domaine de liaison à l'ADN et un domaine d'oligomérisation, impliqué dans la tétramérisation de la protéine nécessaire à son activité transcriptionnelle), elles possèdent en effet un prolongement du domaine C-terminal, absent de la séquence de p53, et qui semble impliqué dans leurs propriétés propres. La maturation des transcrits de p73 et p63 donne lieu à différents splicing-

variants (6 pour p73 et au moins 3 pour p63) en C-terminal, dont les fonctions transcriptionnelles et le pattern d'expression sont différents. De plus, les formes les plus longues des deux protéines présentent un domaine SAM (Sterile Alpha Motif), commun à de nombreuses protéines impliquées dans le développement. Ce domaine est souvent muté dans des syndromes humains impliquant p63, ce qui laisse présager un rôle important dans la régulation de l'activité de p63 et p73. Par ailleurs, des formes tronquées en N-terminal ont été décrites dans la souris : ces formes sont nommées ΔN, elles ne possèdent pas le domaine de transactivation, au contraire des formes longues (TA). Ainsi, ΔNp73 et ΔNp63 agissent dans la souris comme dominants négatifs des fonctions pro-apoptotiques de TAp73 et TAp63. Il a été établi par la suite que ΔNp73 et ΔNp63 sont transcrites à partir d'un second promoteur, localisé dans le troisième intron de la forme longue.

L'étude présentée ici décrit la première caractérisation de la forme ΔNp73 humaine, la régulation de son expression et de son activité. De même que ΔNp73 murine, la forme humaine inhibe les fonctions pro-apoptotiques de TAp73 et p53, via interactions protéines/protéines ou compétition pour les sites de liaison sur les promoteurs cibles. De plus, la présence d'un élément de réponse à p53 situé dans le promoteur de ΔNp73 caractérise une boucle de régulation négative qui s'ajoute à la boucle de régulation MDM2/p53. ΔNp73 agissant comme un oncogène, il semble donc que le ratio ΔN/TA ou ΔN/p53 soit fondamental à l'équilibre cellulaire et que sa dérégulation puisse être impliquée dans la formation tumorale, ce ratio pouvant être contrôlé au niveau transcriptionnel ou post-traductionel de la protéine ΔNp73. L'étude du promoteur de ΔNp73 a mis en évidence de nombreux éléments de réponse à différents facteurs de transcription. De récents travaux ayant associé une augmentation de ΔNp73 à certains types de neuroblastomes, nous nous sommes particulièrement intéressés à N-Myc, facteur de transcription également amplifié dans certains neuroblastomes. Nous n'avons pu toutefois mettre en évidence une activation transcriptionnelle directe de ΔNp73 de la part de N-Myc, mais d'autres éléments de réponse restent encore à caractériser, notamment NFκB et p300, tous deux impliqués dans la régulation de l'apoptose. Par ailleurs, l'étude de modifications post-traductionnelles de ΔNp73 a mis en évidence une rapide dégradation de la protéine après dommages à l'ADN induits par rayons Ultra-Violets ou traitement par drogues, libérant ainsi p53 et TAp73 de son inhibition et permettant ainsi l'apoptose ou l'arrêt du cycle cellulaire. De plus, notre étude a mis en évidence une très brève hémie-vie de la forme ΔN par rapport aux formes contenant le domaine de transactivation. Ainsi, le ratio ΔN/formes longues pourrait également dépendre d'une fine régulation de la dégradation des deux protéines.

INTRODUCTION

An organism is composed of billions of cells and the division of each of them leads to DNA duplication errors. In the majority of cases, the DNA polymerase itself is able to detect and eliminate the mutations. Moreover, every day, cells are exposed to exogenous and endogenous stress which can act on cell metabolism (for example via the Reactive Oxygen Species) and induce apoptosis. But stress (UV, chemical agents, metals) can also damage DNA and cause modifications of the molecule, as, for example, the thymine photo-dimerization. The control machinery of the cell is activated and the process of reparation or the death of the cell, if DNA damage is too important, can occur. If the control of DNA damage or the repairing machinery of the cell fails, a modification in the genetic sequence is introduced : in somatic cells it can lead to cancer and in germ cells to genetic disease of the next generations. One estimate is that a mammalian genome undergoes about 100,000 modifications per day, each bearing a probability of residual damage. Fortunately, only a small part of the genome is a coding region, and nearly 97% can be mutated without any effect. Moreover, the state of each cell (proliferation, differentiation, quiescence) is dependent on signals received through cell-cell and cell-matrix communication, and a tumoral cell has to accumulate mutations, first to escape to this control and then to be positively selected to proliferate and migrate in the organism. Even if at the clinical level cancer is a large diffused disease, cell transformation is a relatively rare event compared to the high level of mutations an organism has to repair every day.

I. Control of DNA damage.

In order to avoid the risk of maintaining a cell dangerous for the other ones, and also for the whole organism, cells have developed control pathways which lead, if the genetic information is damaged, either to cell-cycle arrest and repair of DNA, or to cell death. They have evolved surveillance mechanisms that monitor the structure of chromosomes and coordinate repair and cell-cycle progression. In addition to controlling cell-cycle arrest, these pathways have been shown to control the activation of DNA repair pathways (Cortez et al. 1999; Lim et al. 2000; Gatei et al. 2000; Zhao et al. 2000; Wu et al. 2000), the composition of telomeric chromatin and the movement of DNA repair proteins to sites of DNA damage (Martin et al. 1999; Mills KD et al. 1999), activation of transcriptional programmes

(Elledge 1996), telomere length (Naito et al. 1998; Ritchie et al. 1999) and, in some cell types for reasons not fully understood, induction of cell death by apoptosis (Lowe et al. 1993; Clarke et al. 1993; Xu Y and Baltimore 1996; Hirao et al. 2000).

A. The DNA damage checkpoints.

1. General organization.

The DNA damage response is a signal transduction pathway consisting of sensors, transducers and effectors (**Fig.1**). Although we refer to this as a pathway, it is more accurately described as a network of interacting pathways that together execute the response. The identities of the sensors are not well known. Much is known about the signal transducers, which are composed of four sets of conserved proteins with recognizable motifs. One class is composed of phospho-inositide kinase (PIK)-related proteins which include ATM (Ataxia Telangectasia Mutated) and ATM-Rad3-related (ATR) in mammals and their homologues in budding and fission yeast. These proteins are central to the entire DNA damage response. Downstream of these proteins are two families of checkpoint kinases (CHK), the Chk1 and Chk2 kinases, and their homologues. These kinases carry out subsets of the DNA damage response in mammals and are targets of regulation by ATM and ATR kinases. The fourth conserved family is the BRCT-repeat containing proteins, which include budding yeast Rad9 and fission yeast Crb2. No clear human orthologues of these proteins exist in mammals, but BRCA1 and 53BP1 are possible candidates. The precise roles of these proteins and where exactly they function with respect to the PIKs are not understood.

Below this level of signal transduction are the effectors that execute the functions of the DNA damage response. These include substrates of both PIK and CHK kinases and proteins involved in DNA repair, transcription regulation and cell-cycle control, such as BRCA1, Nbs1, p53 and Cdc25C. Depending on the context, certain molecules such as BRCA1 may have multiple functions in this signal transduction pathway.

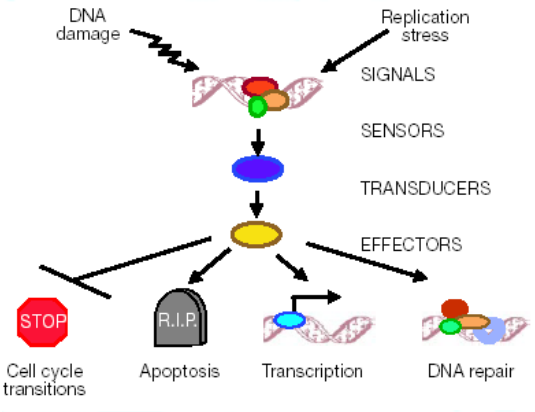


Figure 1. A simplified view of the general outline of the DNA damage response signal-transduction pathway. (Zhou and Elledge 2000)

2. Sensors.

The proteins that initially sense the aberrant DNA structures, and initiate the signaling response, are currently unknown. Owing to their ability to bind and be activated by DNA strand breaks, poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PK) have long been proposed as DNA damage sensors. However, genetic evidence indicates that these proteins are not activators of the global DNA damage response (Wang ZQ et al. 1995; Jimenez et al.1999). In place of these molecules, a group of four conserved proteins in yeast have emerged as candidates because they share some of the properties expected for sensors, including an essential genetic role in the activation of the DNA damage response pathway and the potential to interact with nucleic acids. In *Schizosaccaromyces pombe*, three of these proteins, Rad1, Rad9 and Hus1, are related in structure to PCNA (Proliferating Cell Nuclear Antigen), a repair and replication factor. Modeling suggests that they may form a doughnut-like heteromer like PCNA, and in principle could be loaded onto damaged DNA just as PCNA is loaded onto primed DNA (O'Connell et al. 2000). The human homologues of these proteins form a DNA damage responsive complex (Volkmer 1999). Consistent with yeast studies, inactivation of mouse Hus1 results in impaired responses to genotoxic stress (Weiss et al. 2000).

Alternative proteins have been suggested as candidate sensors, one of which is the breast cancer protein BRCA1. Mouse cells lacking BRCA1 exon 11 are unable to arrest the cell cycle in G2, suggesting a possible defect in sensing or signaling (Xu X. et al. 1999). BRCA1 is part of a large complex named BASC (BRCA1-associated genome surveillance complex) that contains ATM, the Nbs1-MRE11- RAD50 complex, mismatch proteins (MSH2/6 and MLH2), and the Bloom's helicase (BLM) (Wang Y. et al. 2000). This model is speculative, but it is interesting that each of these proteins has the ability to recognize aberrant DNA structures and could thus be involved in transmitting the presence of these structures to ATM and BRCA1. Interestingly, several of these proteins are also substrates of ATM, suggesting that they may be targets of regulation as opposed to directing signal transduction. At present, it is impossible to distinguish between a sensory role and an effector role for any of these proteins, and it is plausible that the activity of an entire complex must be intact to properly sense and respond to damage.

3. Transducers.

In contrast to our knowledge of damage sensors, our understanding of signal transducers is more advanced (**Fig.2**). Two related and conserved proteins, ATM and ATR, are central components of the DNA damage response (Elledge 1996). ATM and ATR are protein kinases structurally related to the PI(3)K family members. The function of ATM is well understood. Cells from Ataxia Telangectasia patients have mutations in ATM and are defective in several responses to IR including G1 arrest (Kastan et al. 1992), reduction in DNA synthesis (Painter and Young 1980) and G2 arrest (Paules et al. 1995). ATM plays an important part in the response to IR, controlling the initial phosphorylation of several key proteins such as p53, Mdm2, BRCA1, Chk2 and Nbs1 after DNA damage. These proteins are still phosphorylated in γ-irradiated AT cells, but with delayed kinetics, indicating that additional pathways respond to IR. Whereas AT fibroblasts are very sensitive to IR, they show little sensitivity to ultraviolet radiation, alkylating agents or inhibitors of DNA replication.

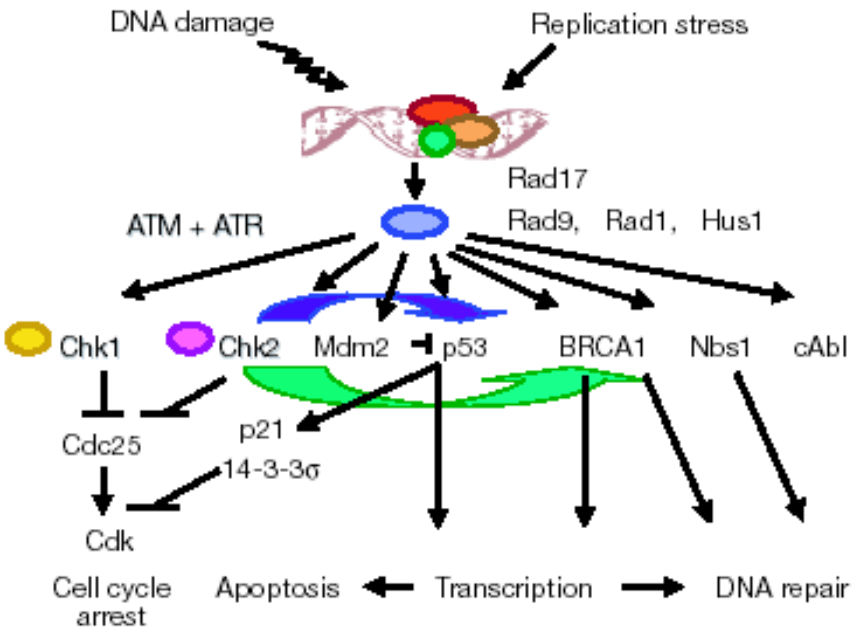


Figure 2. Organization of the mammalian DNA damage response pathway. Although the general organization of the pathway is correct, some details are omitted, especially concerning the relationship between the ATR/ATM and Hus1/Rad17/Rad9/Rad1 proteins, which may participate in mutual regulation. (Zhou and Elledge 2000)

Precisely how these kinases are controlled in response to various stimuli is unknown. ATM kinase activity can be activated by DNA damage *in vivo* (Canman et al. 1998; Banin et al. 1998; Khanna et al. 1998). Direct activation by DNA is less well established. Some studies report that there is no stimulation by DNA *in vitro* (Chan et al. 2000; Banin et al. 1998), others have found that small amounts of purified ATM bind to and are activated by DNA with DSBs (Double Strand Breaks) *in vitro* (Smith et al. 1999). It is not clear, however, whether ATM alone retains the ability to bind DNA or whether other proteins complexed with it are required. ATM may ultimately resemble DNA-PK, which by itself has a low affinity for DSB-containing DNA, but which acquires a significantly enhanced affinity in the presence of a DNA end-binding factor, the Ku 70/80 complex, which loads DNA-PK

onto DNA. Proteins to which ATM is bound are candidate sensors whose identification will be essential for the molecular explanation of ATM activation and DNA damage recognition. Similar activation by damage has not been observed for ATR. However, ATR is likely to be regulated in some fashion because it controls the late phosphorylation of p53 in response to IR (Tibbetts et al. 1999; Lakin et al. 1999; Hall-Jackson et al. 1999) and the ultraviolet-induced phosphorylation of Chk1 (Liu et al. 2000). Thus, it is likely that ATR activation is different from that of ATM and may involve a type of substrate accessibility activation that is not readily recapitulated in vitro. For example, sensor proteins may become activated and recruit substrates to ATR.

B. DNA damage response pathway.

1. Apoptosis.

a) General mechanism.

Apoptosis is an active, programmed cell death, which allows a cell to commit suicide without damaging the neighboring ones. Apoptosis has been also described in unicellular organisms, and even in this case the whole cellular population is important and influences the cell fate (Ameisen 2002). Programmed cell-death involves cell shrinkage, plasma membrane blebbing with partial maintenance of impermeability, mitochondria outer membrane permeabilization, nuclear chromatin condensation and genomic DNA fragmentation, cytoskeletal modifications, and segmentation of the cell into apoptotic bodies. It also involves the expression of various death signaling molecules, such as phosphatidyl-serine, on the outer side of the plasma membrane, that allows neighboring cells to rapidly ingest the dying cell, usually long before it has completed self-dismantling (Savill and Fadok 2000), preventing any reversal of the self-destruction process and leading to the swift elimination of the apoptotic cells. Several of these features of apoptosis contribute to the fact that programmed cell death, in contrast with passive and chaotic necrotic death (that involves cell swelling, plasma membrane rupture, and cell content spilling), usually induces no lesions in the organ and tissue in which it occurs.

With few known exceptions, the terminal apoptotic programme of mammalian cells depends on the activation of intracellular caspases and their modification of protein substrates within the nucleus and cytoplasm

(Hengartner 2000). Two major processes lie immediately upstream of these effector events.

The first is the activation of the receptor mediated death-signaling pathways that ultimately trigger caspase-8 and are exemplified by the interaction of CD95 (Apo-1/Fas) with its ligand (Nagata and Golstein 1995; Krammer 2000). Caspase 8 can in turn activate the mitochondrial pathway, activating Bid, a pro-apoptotic protein (see below).

The second originates from mitochondria, which are central targets for intracellular oxidative stress. This pathway can be profoundly influenced by both pro-apoptotic and anti-apoptotic members of the Bcl-2 family (B-cells Lymphoma 2). The pro-apoptotic proteins Bax, Bad, Bid, Bik and Bim contain an α-helical domain (the Bcl-2 Homolog domain 3, or BH3) that fits the hydrophobic BH3 binding pocket on the anti-apoptotic proteins Bcl-2 and Bcl-X_L, forming heterodimers that block the survival-promoting activity of Bcl-2 and Bcl-X_L. The pro-apoptotic proteins act at the surface of the mitochondrial membrane to induce permeability transition (PT). PT constitutes the first rate-limiting event of the common pathway of apoptosis. Upon PT, apoptogenic factors leak into the cytoplasm from the mitochondrial intermembrane space. Two such factors, Cytochrome C and AIF (Apoptosis Inducing Factor), begin a cascade of proteolytic activity that ultimately lead to DNA fragmentation. In the presence of dATP, Cytochrome C complexes with and activates Apaf-1 (Adaptor molecule apoptosis Protease Activating Factor 1). Activated Apaf-1 binds to caspase 9, and this form the apoptosome complex (Apaf-1, Cytochrome C, dATP and Caspase 9), where caspase 9 is activated (Kroemer and Reed 2000). This begins a caspases cascade ultimately resulting in DNA fragmentation. Thus, the relative abundance of pro- and anti-apoptotic Bcl-2 family proteins determine the susceptibility of the cell to programmed cell death. Numerous survival, or pro-apoptotic factors regulate this ratio, either at a transcriptional level (p53), or by post-translational modifications (phosphoinositide 3-kinase (PI(3)K) and Akt (Korsmeyer 1999)). For example, the protein kinase Akt/PKB can be activated by various growth factors and its activity can be blocked by PTEN. Akt functions to promote cell survival through two distinct pathways : it inhibits apoptosis by phosphorylating Bad, which then interact with 14-3-3 and de-associates from Bcl-X_L, allowing for cell survival. or alternatively, it activates IKK-α that ultimately leads to NF-κB activation and cell survival.

A third pathway, the autophagy, starts to be studied in mammalian cells but is much better understood in yeast. In case of amino acid depletion for

example, cells start to digest slowly their components in lysosomes until the environment becomes more favorable. It seems that in some cases mammalian cells activate this “primitive way of dying”.

Apoptosis is not only a response to cell injury, but is also responsible for the modeling of numerous embryonic structures. In fact, apoptosis participates to the sculpting of the form of the embryo : during embryogenesis, numerous cells are produced in excess and are eliminated to shape the organs and the whole embryo. The most classical example is the interdigital space, vestige of the evolution, which disappear to form the definitive hands and feet. Another example is the large excess of neurons that will die by competition for their innervating targets, which allow the great plasticity of the whole nervous system. It appears more and more clearly that a cell is always “ready to commit suicide”, and this doesn’t happen till the neighboring ones send it life signals.

b) Apoptotic and survival pathways after stress.

Radiation damage triggers multiple stress and apoptotic pathways dependent on the cell type involved (**Fig.3**). Stress signals generated outside the nucleus include activated mitogen-activated protein kinase (MAPK) cascades, extra-cellular signal-regulated protein kinase (ERK), JNK and p38 and protein kinase C (PKC) (blue arrow). Transcription factors are an important target of MAPK cascades, ERK activation tends to favor survival, whereas JNK activation assists cell death. The RedOx-sensitive transcription factor NF-κB also translocates to the nucleus after its activation by reactive oxygen species (ROS). The activity of this transcription factor is generally associated with protection from apoptosis, but it has been also shown that its activation correlates with the ability of p53 to induce apoptosis (Ryan et al. 2000). Expression levels of several membrane death receptors might be augmented by stabilized p53 (red dashes). These outcompete decoy receptors, leading to the activation of caspases and an additional caspase-dependent pathway that loops through the mitochondrion (M) via Bid. Stabilized p53 also increases the concentration of Bax while diminishing the level of Bcl-2, thus favoring the disruption of mitochondrial membranes and, ultimately, the activation of caspases. The non-receptor tyrosine kinase, c-Abl, has dual roles in the cytoplasm and in the nucleus. The nuclear version is activated by ATM and can stabilize p53. Several pro-apoptotic activities have been suggested for c-Abl, although it is probably fair to say that many of these are still speculative. An important survival pathway (green arrow) is

the protein-kinase-B-mediated inactivation of Bad, which is inhibited by cytoplasmic c-Abl.

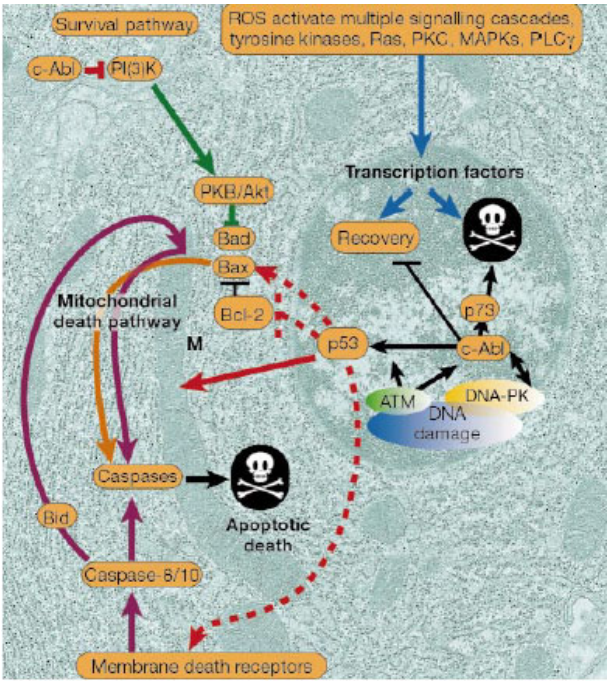


Figure 3. Apoptotic and survival pathways. (Rich et al. 2000)

4. DNA damage response and repair are interacting networks.

Recent observations have made it clear that the response to DNA damage in mammals is not limited to decisions on cell-cycle arrest and apoptosis, but is intimately involved in controlling repair itself. DNA repair pathways consist of an intricate network of repair systems that each target a specific subset of lesions (**Fig.4**). Much of DNA repair is constitutive, but a number of regulatory connections between the DNA damage response pathway and DNA repair have emerged. First, in yeast and mammals, a large

number of genes involved in DNA repair are transcriptionaly induced in response to DNA damage in a DNA damage response pathway dependent manner, suggesting that many facets of repair are enhanced (Elledge 1996; Jelinsky and Samson 1999). Second, fibroblasts lacking p53 have been shown to be defective in global excision repair of cyclobutane dimers. The p48 gene, which is mutated in Xeroderma pigmentosum group E cells, is induced by DNA damage in a p53-dependent fashion (Hwang et al. 1999), possibly explaining p53's role in excision repair. Recently, a new nuclear localized subunit of ribonucleotide reductase, p53R2, was found to be induced by p53 in response to DNA damage. Blocking p53R2 expression increases cell killing by a variety of DNA damaging agents (Tanaka et al. 2000), supporting a functional role for p53R2 in DNA repair. Regulation of ribonucleotide reductase through the DNA damage response pathway represents a conserved strategy employed by the DNA damage response kinases to facilitate repair (Elledge 1996). Together these results challenge the long-held notion that p53 functions mainly to induce apoptosis and suggest that p53 also promotes cell survival in response to DNA damage under certain circumstances, as we will see in detail below.

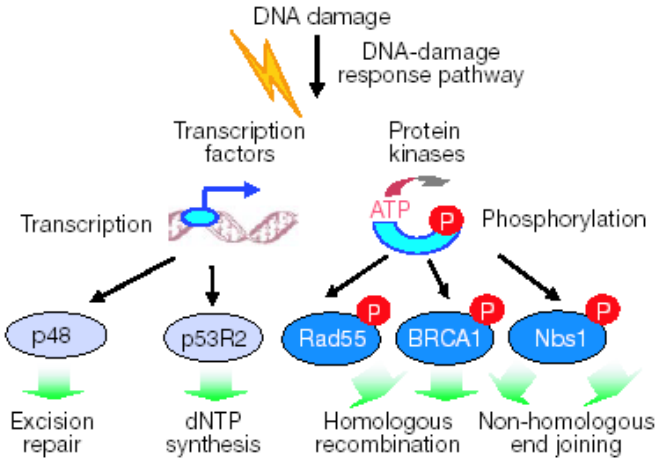


Figure 4. Interactions between the DNA damage response pathway and DNA repair networks. The regulatory connections include transcriptional upregulation of repair proteins such as p48 and p53R2 after DNA damage, and phosphorylation of repair proteins such as BRCA1, Nbs1 and Rad55 after DNA damage. (Zhou and Elledge 2000)

a) Double-strand break (DSB) repair.

A DSB is potentially lethal. Two competing repair processes called homologous recombination and non homologous end-joining (NHEJ) target DSBs (Karran 2000) (**Fig.5**). Homologous recombination uses a sister chromatid or homologue to patch up the damage, whereas NHEJ is less accurate and simply joins DNA ends together. Variations of each process exist, most importantly in the use of conservative or non-conservative homologous recombination, which, as the name suggests, have different mutagenic potentials. NHEJ and homologous recombination are often described as the dominant repair pathways for mammals and yeast respectively. Despite its inaccuracy, mammals seem to favor NHEJ as their repeat-ridden genomes make sequence alignment tricky. But it is now known that vertebrates are also proficient at homologous recombination (Liang et al. 1998), prompting a major reassessment of the value of this process to mammalian repair. The mechanics of NHEJ entails the binding of Ku heterodimers to DNA breaks, protecting them from degradation and stabilizing the lesion. Ku then recruits the catalytic subunit of DNA-PK (DNA-PKcs) to activate the DNA-PK holoenzyme. The formation of this activated nucleoprotein complex promotes rejoining by a DNA ligase IV–XRCC4 heterodimer (XRCC4 denotes the X-ray cross-complementation group containing a deletion of the XRCC4 gene product). For the particular case of homologous recombination shown, hRAD52 is recruited to the DNA break, followed by invasion of the intact sister chromatid by hRAD51 to generate a recombination intermediate. As the sister chromatid acts as a template, repair must take place in late S or the G2 phase of the cell cycle. The breast-cancer susceptibility gene BRCA1 product co-localizes with hRAD51 and promotes homologous recombination, perhaps to discourage the less accurate NHEJ process. There is also considerable interest in the Mre11–Rad50–NBS1 complex, which performs the nucleolytic processing of DSBs and is also implicated in cell-cycle checkpoints through ATM.

b) Single-strand break repair.

Single-strand repair is determined by the site and nature of the break. Nucleotide excision repair (NER) is used to excise bulky lesions (**Fig.6**), such as pyrimidine dimers, that distort the DNA helix. Two NER machines repair the inactive (the global mechanism) and active portion of the genome; RNA polymerases have a major role in the latter. For either complex, lesion recognition is followed by excision of the damaged DNA (steps 1–3 and 1–4 in the respective panels) so that re-replication can occur.

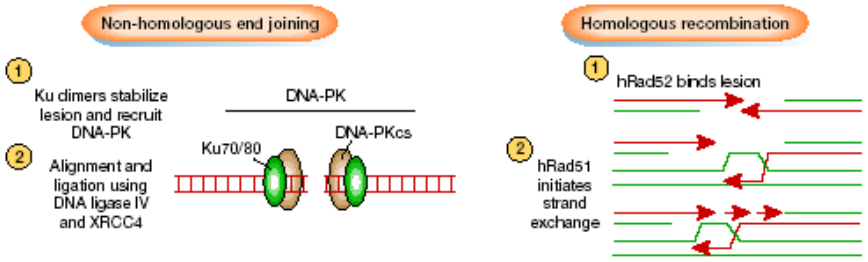


Figure 5. Repairing double-strand breaks. (Rich et al. 2000)

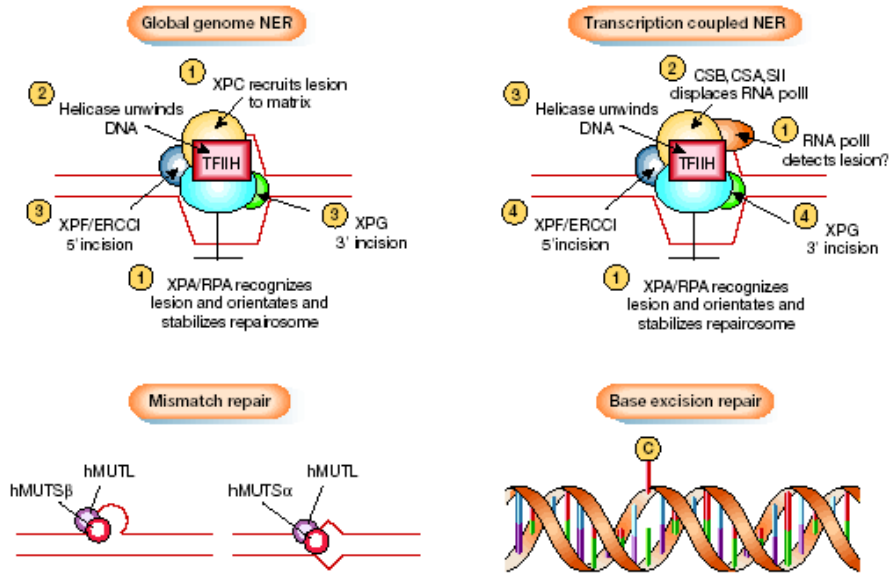


Figure 6. Repairing single-strand. (Rich et al. 2000)

Mismatch repair (MMR) detects several types of single-base mismatches in addition to more complicated loops or deletions (Kolodner 1999). Current interest in this process derives from the identification of defective MMR genes as the causative agents of hereditary non-polyposis cancer. Various combinations of hMUTS and hMUTL heterodimers recognize each class of lesion to recruit repairosomes. ATP hydrolysis facilitates either translocation/looping of the DNA or the conversion of hMUTS to a sliding clamp that activates and recruits repair proteins including hMUTL complexes, polymerases-δ/ε, exonucleases and replication factors. Finally, base excision repair removes small lesions such as alkylated and methylated bases. This is an ancient repair process that counteracts the natural instabilities of DNA as well as those posed by environmental genotoxins (Krokan 2000). In the example shown, the damaged base is literally swung out of the helix and into the 'pocket' of a correcting enzyme (yellow ball), which snips it from the helix. The abasic site can be processed by APE1 endonuclease before DNA polymerase β inserts the correct nucleotide and XCC1/ligase III seals the nick. These proteins may be orientated on PARP. 'Short patch repair' is used in this instance, although 'long patch repair' is available for gaps of two to eight nucleotides.

In some cases large complexes of proteins must sequentially assemble over the lesion. This raises the critical question of how DNA damage detectors should be distributed in a manner that allows them to survey the entire genome. Although the 'active' nucleotide excision repair (NER) repairosome can tether itself to complexes that naturally navigate the DNA thread, not all repair processes are tied to transcription or replication. An attractive solution would be to corral repair proteins at various nuclear foci for release under conditions of genotoxic stress.

C. A major role for p53 after DNA damage and the emergence of its two new relatives.

Cells differ in their response to DNA damage : their sensibility to ionizing radiation or drug treatment usually decrease when they differentiate. For example, the post-replicative epithelial cells of the adult intestinal crypt are resistant to apoptosis in response to ionizing radiation and many other DNA-damaging agents, but replicative cells of the same lineage, just a few hours earlier in their life history and one cell position deeper in the crypt, are

highly sensitive to both radiation-induced and drug-induced apoptosis (Bach et al. 2000).

The final decision to initiate apoptosis rather than cell-cycle arrest or a failure to respond by either route is likely to be conditioned by the magnitude and duration of the damage stimulus. It will also reflect the damaged cell's replicative status, its recent history as demonstrated by the availability of apoptosis effectors, and even its position, because the local growth factor environment expresses proximity to neighboring cells and to basement membrane. **Figure 7** illustrates the extreme complexity of the ATM checkpoint and shows a major role for p53 activated by DNA damage on the different steps of cell cycle, as will be detailed below. p53 is able to lead the cell either towards cell-cycle arrest and DNA repair or to apoptosis. The mechanism by which the decision is taken is not yet fully understood, but the activation, regulation and action of p53 have been well studied. The fact that p53 is mutated in 50% of cancers and that its sequence is conserved from mollusks to human led to call it "The Guardian of the Genome". The regulations of its action are extremely complex and a lot of partners have been identified : they allow p53 either to block the cell cycle or to induce cell death in case of DNA damage.

However, for almost 20 years, it seemed, surprisingly, that p53 was alone to play such an important role in the cell. In fact, many of tumor suppressors are part of gene families, which allow the cell to compensate a possible mutation of the gene by the redundancy of function of the other family members. Any attempt to find p53-related genes failed, until 1997, when a human homolog of p53 has been identified and called p73. The year after, another member has been found (p63), and it seems now that the family is complete.

A very recent work explored the implication of p63 and p73 on DNA damage-induced apoptosis in mouse embryo fibroblasts deficient for one or a combination of the p53 family members. It appeared that the combined loss of p63 and p73 results in the failure of cells containing functional p53 to undergo apoptosis in response to DNA damage (Flores et al. 2002), suggesting a strong inter-dependence between the 3 family members.

In fact, p73 and p63 are able to activate some p53 targets and to induce apoptosis (**Fig.7**), but in the light of p73 and p63 study, p53 appears more and more different from its two relatives : the genes TP73 and TP63 give rise to numerous proteins isoforms which may play very different roles from differentiating factors and apoptosis inducers to apoptosis inhibitors, which makes the story more and more complex, as we will develop now.

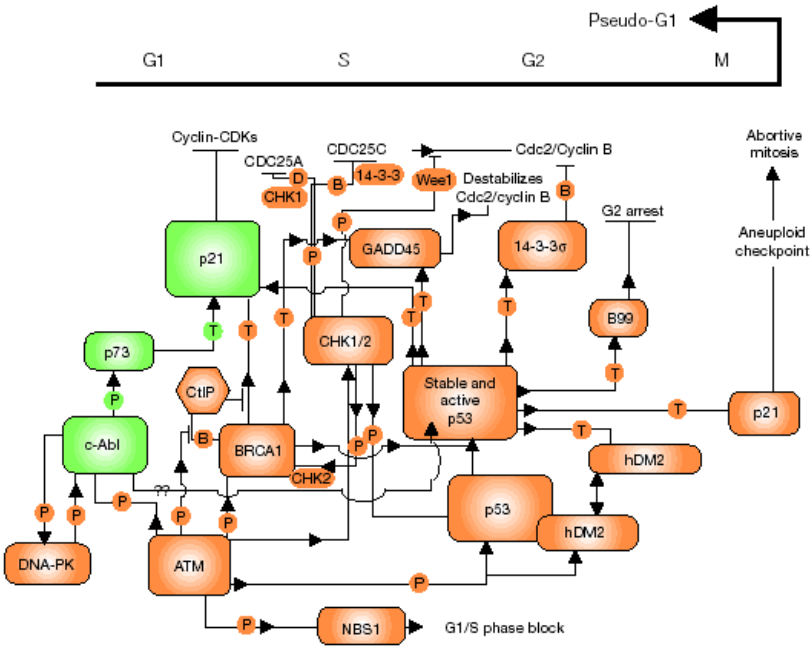


Figure7. ATM, checkpoints and the cell cycle.
DNA damaged by ionizing radiation can be sensed by ATM, triggering a cascade of downstream pathways to arrest the cell cycle. ATM-proximal events are phosphorylation reactions (denoted by P) that can lead to downstream transactivation events (T), degradations (D) or inhibitory blockades (B). p21 can block the G1/S transition and prevents aneuploidy. Multiple proteins transactivated by p53 block the S/G2 transition. Green boxes mark an auxiliary pathway that uses p73 to activate p21. S-phase blocking is also achieved by the phosphorylation of CDC25C by CHK, resulting in its cytoplasmic sequestration (by 14-3-3). Similarly, Cdc2 and cyclin B are inhibited by 14-3-3s. (Rich et al. 2000)

II. p73 : member of the p53 transcription factors family.

The report of the first p53 homolog, p73, and the fact it was located in a long-suspected tumor suppressor locus (1p36, a region frequently deleted in neuroblastoma), was met with great excitement and anticipation. The hallmark features of the p53 protein (an acidic, amino-terminal transactivation domain, a core domain for DNA binding and a carboxy-terminal oligomerization domain) are shared by p73. Notably, the DNA-binding domain, wherein nearly all cancer-associated p53 mutations are found, show over 60% identity with that of p53.

Although significant homology can suggest a conservation of function, we cannot simply assume these relationships. While the p53 family members have established roles in tumorigenesis, DNA damage and/or development, many functions of these proteins are still unknown or doubtful, and p73 and p63 develop more and more their own identities.

However, the nearly 20 years of p53 study allow to understand better the identities and differences between p53 and its two new relatives.

A. p53 and the tumoral process.

1. A central role in tumor development.

p53 has been identified in 1979 in cells transformed by SV40 virus (Simian Virus 40), and was first thought to be an oncogene, as it was able to bind to antigen-T and was stabilized by this binding. p53 has been characterized as a tumor suppressor gene instead of an oncogene only 11 years after, when it has been clear that T-antigen bound to p53 inhibits its transcriptional activity and was shown that the originally cloned p53 cDNAs used in the early experiments contained dominant negative missense mutations within a conserved region of p53, important for both the conformation and biological activity of the protein.

p53 protein is encoded by a tumor suppressor gene, which is mutated in more than 50% of human cancers (Soussi & Béroud 2001). Even in cancer types in which TP53 mutations are rare, p53 function is indirectly abolished either by nuclear exclusion (neuroblastoma), interaction with a viral protein (cervical cancer), interaction with overexpressed MDM2 protein (sarcoma) or inactivation of $p19^{ARF}$ (Moll et al. 1996; Oliner et al. 1993; Crook et al. 1992). There are a few tumours in which TP53 mutations have never been

detected, such as testicular cancer and melanoma; but in melanoma the apoptotic pathway that is induced by p53 in response to chemotherapeutic agents is affected by alterations in the APAF gene, which acts downstream of p53 (Soengas et al. 2001).

The importance of these observations was underscored by the finding that mice transgenic for expressing mutant p53 alleles (Lavigueur et al. 1989) or homozygously defective for p53 (Donehower et al. 1992; Harvey et al. 1993; Purdie et al. 1994; Jacks et al. 1994) develop normally but they have a high incidence of tumors, especially of lymphoid tissue.

Moreover, the human TP53 mutation leads to a dominant autosomic disease, the Li-Fraumeni syndrome, which enhances dramatically predisposition to sarcoma, breast cancer or brain tumors (Malkin et al. 1990). In individuals who have Li–Fraumeni syndrome but lack TP53 mutations, Bell *et al.* have described germ-line alterations of the CHK2 kinase, which activates p53 after DNA damage (Bell et al. 1999). Cells from patients with the radiosensitive and cancer-prone disease Ataxia Telangectasia show radioresistant DNA synthesis and a reduced or delayed γ–radiation-induced increase in p53 protein levels. This is due to an inactivating germ-line mutation in ATM, which activates p53 in response to irradiation, as we have seen before (Rotman et al. 1999).

2. p53 domains : structure and function

The human TP53 gene is situated on the chromosome 17, in position p13.1 and is composed of 11 exons. Alternative splicings have been described, but their biological relevance has not been established yet (Flaman et al.. 1996).

The p53 has been conserved during evolution (Soussi et al. 1987). Through cross-species comparison of amino acid sequences, the p53 proteins showed the existence of five highly conserved regions within the amino acid residues 13-23, 117-142, 171-181, 234-250 and 270-286 (Soussi et al. 1990; Soussi and May 1996) (**Fig.8**). These regions, termed domains I-V, were expected to be crucial for the p53 functions. The more recent data of the literature confirm this view (Soussi and May 1996).

The acidic N-terminal transcriptional domain (1 to 62) allows p53 to recruit the basal transcriptional machinery, including the TATA box binding protein (TBP) and TBP-associated factors (TAF) components of TFIID (Lu and Levine 1995; Thut et al. 1995). It contains two transactivating domains : AD1 and AD2 (Candau et al.. 1997, Zhu JH et al. 1998). AD1 binds to

transcription co-factors, repair or replication proteins. AD2 seems to be responsible for pro-apoptotic genes activation. This N-terminal region is involved in p53 stability and activation.

The Proline-rich region (63 to 91) is composed of five repetitions of the motif PXXP (Walker & Levine 1996). This region may bind to anti-proliferative proteins with SH3 domains, it seems to be essential for p53-dependent apoptosis (Sakamuro et al. 1997) and may be involved in physical interaction with elements of signal transduction pathways, for example c-abl. Although deletion of the proline-rich domain does not affect transactivation of several promoters it does alter transcriptional repression (Venot et al. 1998).

The DNA-binding domain (102 to 292) contains the four conserved regions II-V. It is within this central part that 80-90% of the tumor mutations are found and this region also functions as a protein binding domain interacting with simian virus 40 (SV40) large T antigen (Jenkins et al. 1988). Moreover, it is known that the p53 protein forms tetramers, which are the functional forms (Kraiss et al. 1988; Clore et al. 1994, 1995). X-ray crystallography analysis by Cho and colleagues (Cho et al. 1994) of the crystal structure of the core DNA-binding domain bound to its cognate site has been immensely informative (**Fig.9**). Under these conditions, the structure of the core domain consists of a large β sandwich that acts as a scaffold for three loop-based elements. The sandwich is made up of two antiparallel β sheets containing four and five β strands, respectively. The scaffold anchors the loops and participates in head-to-tail dimerization. The first loop L1 (LSH for loop-sheet-helix) binds to DNA within the major groove. The second loop L2 binds to DNA within the minor groove. The third loop L3 packs against L1 and stabilizes it. The L2 and L3 loops are connected by a zinc atom tetrahedrally coordinated on amino acids Cys176, His179, Cys238 and Cys242. This zinc atom stabilizes the structure of the loops. Such a role of zinc is substantiated by the fact that metal chelating agents inhibit the sequence-specific DNA binding and change its conformation as detected by antibodies (Hainaut and Milner 1993a,b).

The C-Terminal domain (300 to 393) has got several functions. It contains the oligomerization domain, necessary for the p53 transcriptional activity. The structure of this tetramerization domain has been deduced from nuclear magnetic resonance (Clore et al. 1994) and X-ray crystallography (Jeffrey et al. 1995). Tetramerization appears to be required for efficient transactivation in vivo and for p53-mediated suppression of growth of carcinoma cell lines (Pietenpol et al. 1994).

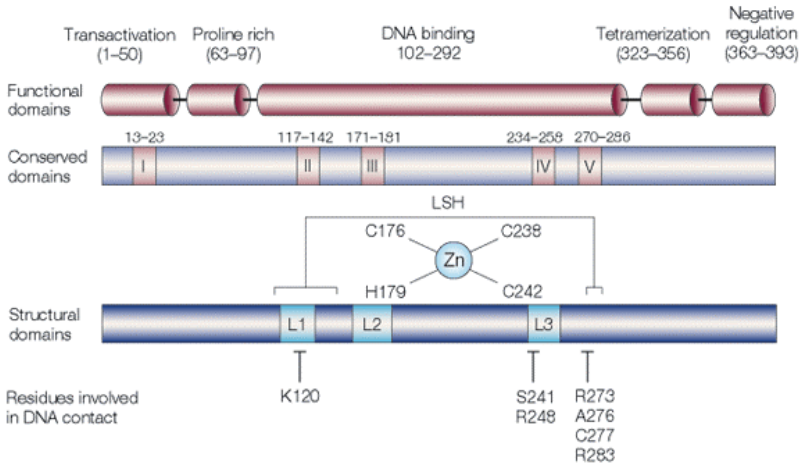


Figure 8. Schematic representation of the p53 protein.
The functional domains, regions of sequence conservation, and structural domains. L1, L2, and L3 indicate loops, and LSH indicates a loop–sheet–helix structure. Tetrahedrally coordinated zinc is necessary for DNA binding.
(Soussi and Beroud 2001)

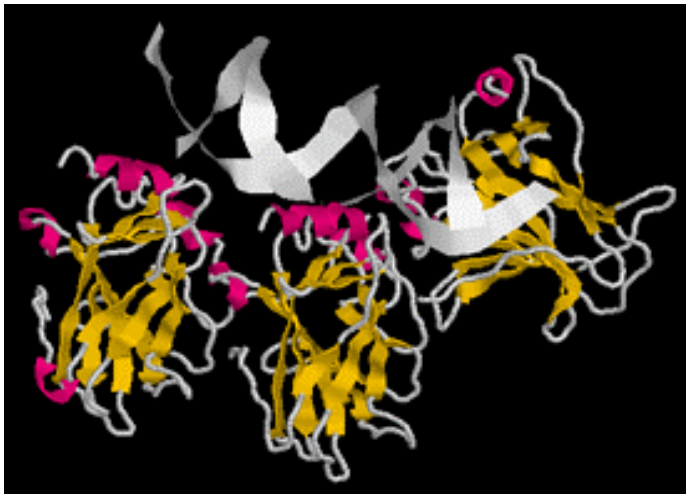


Figure 9. p53 interaction with DNA.
Crystallographic structure of the p53 DNA binding domain in contact with DNA, in a trimeric form.

Moreover, two NLS (Nuclear Localization Signal) and one NES (Nuclear Export Signal) are present and regulate p53 cellular localization. Adjacent to the oligomerization domain (323-356), there is a basic region (amino acids 363-393) which has also been referred as an apoptotic domain (Wang XW et al. 1996), a transcriptional regulatory domain (Wang and Prives 1995) or a DNA damage recognition domain. This extreme C-terminal domain (amino acids 363-393) seems to act as a negative regulator of p53 sequence specific binding, which could hide the DNA binding domain in the non-stressed cells and liberate it after a stress-induced conformational change (Hupp et al. 1992).

3. Regulation of p53 stability.

The p53 protein is found at very low levels in normal, unstressed cells, and presents a short half-life (about 20'). In this way, it doesn't interfere with cell cycle or cell survival in normal conditions of growth. Regulation of protein stability is a common mechanism by which the function of cell growth regulatory proteins is controlled and p53, like many other proteins, is targeted for degradation by the proteasome following ubiquitination. This form of proteolysis involves a system of enzymes that conjugate multiple ubiquitin, a 76 amino acid polypeptide, to lysines in the targeted protein (Varshavsky, 1997). These polyubiquitinated proteins are then recognized and degraded by the proteasome. Attachment of ubiquitin to the proteins destined for degradation depends on three activities : E1, the ubiquitin-activating enzyme; E2, the ubiquitin-conjugating enzyme and E3, the ubiquitin-ligase (**Fig.10**). The ubiquitin-ligases comprise a large and diverse group of proteins, sometimes functioning in large multiprotein complexes, and these are the enzymes that are responsible for determining the substrate specificity of the ubiquitin pathway.

Numerous post-translational modifications of p53 lead principally to a regulation of its binding with its negative regulators : Mdm2 and the Jnk proteins, which are able to induce p53 degradation.

a) Mdm2.

Mdm2 (Murine Double Minute 2) has been identified as the product of the MDM2 oncogene, which is amplified in some types of tumor (Momand et al. 1992; Oliner et al. 1993). It presents at least 40 different transcripts in normal and tumoral cells, due to alternative splicing and to the presence of 2 promoters. The mice MDM2-null are lethal (Jones et al. 1995; Montes de

Oca Luna et al. 1995), but this phenotype is reverted in the double Knock-Out TP53 -/- ; MDM2 -/-, where the mice present the same phenotype as the TP53-null. This strongly suggests that loss of Mdm2 leads to uncontrolled p53 activity. Mdm2 is an E3 ubiquitin-ligase with a C-terminal RING finger domain (Tyers and Willems 1999). It presents both nuclear export and nuclear import sequences, which are necessary for p53 regulation

The negative regulation of p53 through Mdm2 functions in two major ways. Firstly, Mdm2 binds to the N-terminal domain of p53 and thereby inhibits the ability of p53 to stimulate transcription (Momand et al. 1992; Oliner et al. 1993). For example, the coactivating protein TAF(II)D competes with Mdm2 for its binding site on p53 (Buschmann et al. 2001a). Secondly, Mdm2 also plays a central role in the regulation of p53 levels since the oncoprotein targets p53, and also itself, for rapid degradation (Haupt et al., 1997a; Kubbutat et al., 1998). In the nucleus, Mdm2 binds specifically to the p53 N-terminus and induce the ubiquitination of the C-terminal lysines (370, 372, 373, 381 and 382) (Nakamura et al. 2000; Rodriguez et al. 2000), thanks to its E3 ligase activity. p53's NES is inactive when the protein is in the tetrameric form. The ubiquitination of the lysine residues in the C-terminus, allows the NES to be revealed by a conformational change, so that the protein is exported (Honda et al. 1997; Freedman and Levine 1998; Vousden 2000; Lohrum et al. 2001), and in the cytoplasm, p53 is degradated by the proteasome pathway.

Another action of Mdm2 on p53 has recently been described : Mdm2 induces translation of p53 mRNA from two distinct alternative initiation sites, resulting also in the production of ΔN-p53 (also called p53/47), a shorter amino terminal truncated protein lacking the transactivation domain (Yin et al. 2002; Courtois et al. 2002). This translation requires Mdm2 to interact directly with the nascent p53 polypeptide. ΔN-p53 does not contain the Mdm2 binding site, but it is still able to hetero-oligomerize with p53, negatively regulating its transcriptional and growth-suppressive activities. Therefore, Mdm2 regulates the steady-state protein levels of p53 at its synthesis level.

Moreover, it is noteworthy that the two mdm2 promoters present p53 responsive elements, which gives the opportunity for feedback negative control of p53 activity (Wu et al. 1993) (**Fig.11**).

A MDM2 homolog has been recently identified and characterized : MDMX (Shvart et al. 1996). The role of MdmX is not yet fully understood. MdmX is able to bind p53 without targeting it for degradation. It binds to and stabilizes Mdm2 via their C-terminal domain (Sharp et al. 1999) and

protect p53 from the Mdm2-induced degradation (Jackson and Berberich 2000). However, it has been recently described that MdmX is a negative regulator of p53 activity as low levels of MdmX in the cell are correlated to p53 stabilization, increase of UV-induced apoptosis and higher levels of Bax expression (Finch et al. 2002).

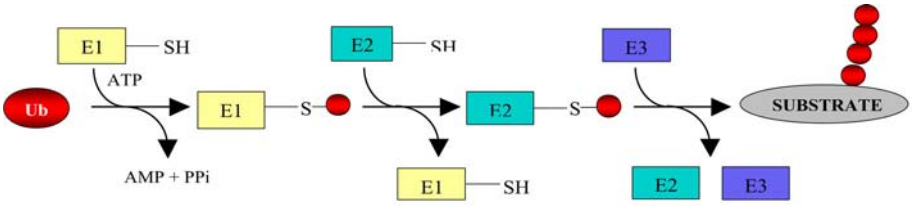


Figure 10. Ubiquitination.
Free Ubiquitin (Ub) is activated in an ATP-dependent manner with the formation of a thiol-ester linkage between E1 and the carboxyl terminus of ubiquitin, Ubiquitin is transferred to one of a number of different E2s. E2 associates with E3, which have or not substrate already bound. In the case of RING E3s, it seems that ubiquitin is transferred directly from the E2 to the substrate.

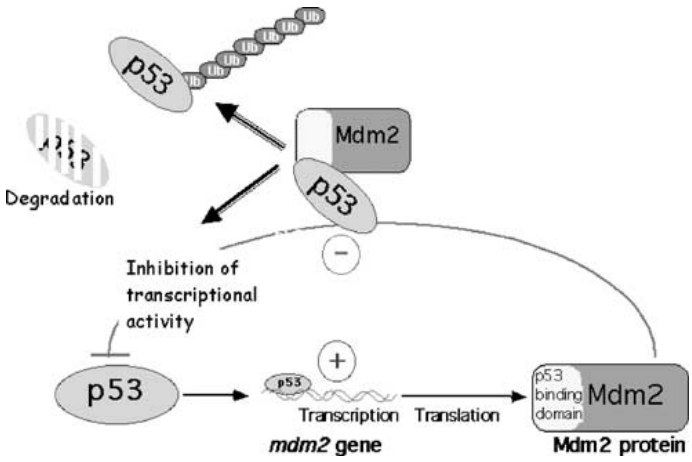


Figure 11. MDM2 regulates p53 activity in a negative feedback loop. (Oren 1999)

b) The JNKs proteins.

Other cellular proteins also appear to contribute to the degradation of p53 in normal cells, and the interaction of JNK (c-Jun NH2-terminal kinase) with p53 has been shown to regulate p53 ubiquitination and stability (Fuchs et al. 1998a). The JNK protein is part of the Mitogen-Activated Protein Kinases (MAPK) family which are involved in signal transduction and are able to induce cell differentiation or apoptosis after stress signals (Ip and Davis 1998). JNK-directed degradation is independent of Mdm2 and does not require the kinase activity of JNK. The mechanism by which JNK functions to regulate p53 stability is not yet understood, although it has been suggested that JNK can function as part of a ubiquitin-ligase (Fuchs et al., 1998a), and is able to phosphorylate p53 on Thr81 in order to activate it after a stress signal, , as we will see below (**Fig.12**). JNK1, 2 or 3 deficient mice do not show the early p53 dependent lethality characteristic of MDM2 deficiency, although this could reflect compensatory activities of the remaining JNK family members.

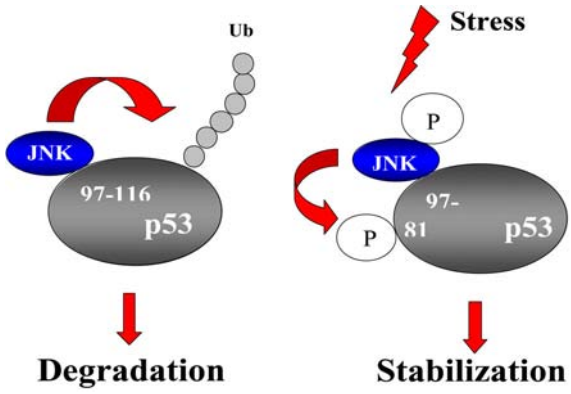


Figure 12. The JNK proteins induce p53 degradation or stabilization.
In a non-stressed cell, JNK induces p53 ubiquitination and thus degradation. After a stress signal, activated JNK phosphorylates p53 on Thr81, which is then stabilized.

c) Other p53 degradation pathways.

It has been recently shown that the HAUSP protein (Herpes virus Associated Ubiquitin Specific Protease) interacts with p53 to induce its de-ubiquitination *in vitro* and *in vivo* (Li M et al. 2002). HAUSP stabilizes p53 even in excess of Mdm2 and is able to induce cell cycle arrest and apoptosis.

The de-acetylase mSin3a interacts with the p53 proline-rich region and protects it against the proteasome dependent degradation, independently of Mdm2 and JNK proteins (Zilfou et al. 2001).

The NADH Quinone Oxydoreductase 1 protein (NQO1), whose expression is activated by p53, is able to stabilize p53 in a distinct pathway. The inhibition of NQO1 increases Ubiquitin-dependent p53 degradation (Asher et al. 2001).

Finally, sequences within the p53 DNA binding domain seem to be involved in an interaction with a fragment of the transcriptional co-activator p300/CBP (Grossman et al. 2003). It has been observed that a p53 mutant defective for binding to p300 is resistant to MDM2-mediated degradation. It seems that, while Mdm2 catalyzes the addition of a single ubiquitin to a cluster of six C-terminal lysines in p53, p300 can polyubiquitinate p53, thanks to an E4 activity, and needs as a substrate the Mdm2-ubiquitinated p53. N-terminal region of p300 interacts with Mdm2 which allows to bring together necessary catalytic and/or regulatory factors needed for p53 ubiquitination. The full length p300/CBP has a second contribution in p53 activation, which will be detailed in the next part.

4. p53 activation.

Different stresses may induce p53 activation : (i) genotoxic stress, such as ionizing radiations, UV or chemotherapeutic drugs which are able to induce DNA damages (Huang et al., 1996; Kastan et al., 1991; Maltzman and Czyzyk, 1984); (ii) non genotoxic stress which may involve the spindle checkpoint (Cross et al. 1995), ribonucleotides starvation (Linke et al. 1996), hypoxia (Graeber et al. 1996), heat shock (Ohnishi et al. 1996), nitric oxide exposure (Forrester et al. 1996) and (iii) finally, oncogenes activation (Sherr, 1998) (**Fig.13**).

Nevertheless, it seems that p53 induction is principally due to a stabilization of the protein and that p53 transcription increase is only a minor event. The activation of p53 in cultured mammalian cells has been correlated with phosphorylation (Ko and Prives 1996), acetylation (Sakaguchi et al.

1998) glycosylation (Shaw et al. 1996), sumoylation (Meek 1998), binding to regulatory proteins and proteolytic removal of the C-terminal domain (Okorokov et al. 1997). The p53 protein is then stabilized and accumulates in the nucleus to transactivate the expression of its target genes, in order to "repair" the cell or induce its death.

An early clue was the observation (Maltzman and Czyzyk, 1984) that wt p53 is stabilized in cells exposed to UV radiation, and a considerable amount of research has since confirmed the central role of DNA damage in mediating the p53-induced growth arrest or apoptosis. Following the DNA damage the p53 protein rapidly accumulates and becomes activated (Kastan et al. 1991; Lu and Lane 1993), probably in response of double strand breaks induced by the genotoxic agents. p53 binds strongly to strand breaks by its C-terminal domain (Nelson and Kastan 1994) and this leads to the stabilization and activation of the protein.

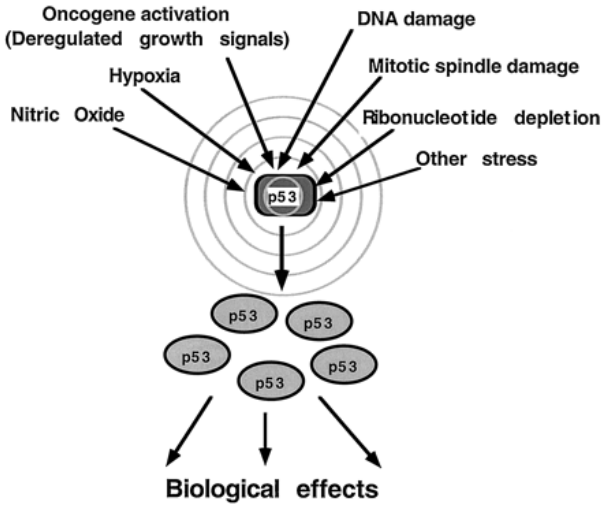


Figure 13. Signals that activate p53.
Activation results in markedly increased overall p53 protein levels and most probably also in qualitative changes in the protein (Oren 1999).

a) Phosphorylation.

With the understanding that p53 degradation is regulated in large part by Mdm2 came the realization that stabilization of p53 is likely to involve mechanisms to protect p53 from Mdm2. One of the most obvious ways is to prevent the interaction between the two proteins, and this could be achieved by modification of either proteins in response to an activating signal (**Fig.14**).

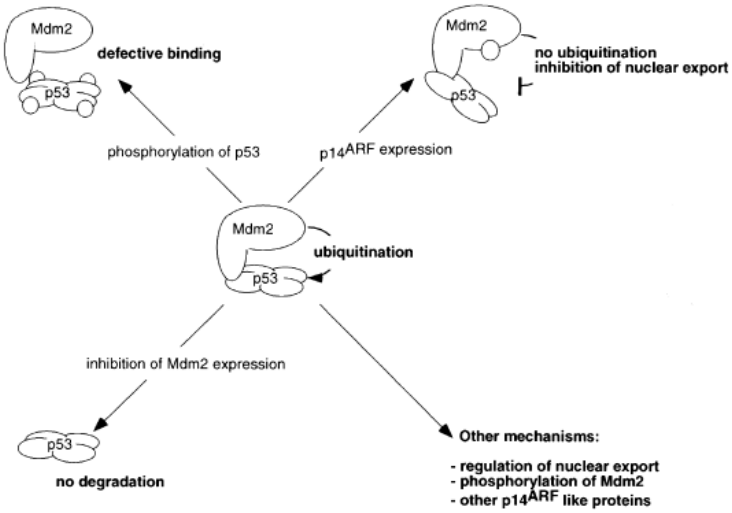


Figure 14. Inhibition of MDM2-mediated degradation can occur through several pathways (Ashcroft and Vousden 1999).

Recently, p53 has been shown to be inducibly phosphorylated at a number of sites after various forms of DNA damage (Meek 1998). A plethora of kinases have been shown to phosphorylate residues within the N-terminus of p53 *in vitro* (See **Fig.2**), including ATM (Ataxia Telangectasia Mutated), ATR (ATM-Rad3-related), DNA-PK, JNK (See **Fig.12**) and CKI (Jayaraman and Prives, 1999). Endogenous p53 has been shown to be phosphorylated at several sites following DNA damage, including serine 15, 20, 33 and 37 (Siliciano et al. 1997; Banin et al. 1998; Canman et al. 1998; Shieh et al. 1999), and there is evidence that ATM and

ATR can phosphorylate serine 15 *in vivo* (Khanna et al., 1998; Tibbetts et al., 1999). Our understanding of the structural requirements for the p53/Mdm2 interaction (Kussie et al., 1996) indicates that phosphorylation within the N-terminus of p53 could impede binding between the two proteins, although the observation that different patterns of phosphorylation occur in response to different stabilizing signals indicates that no individual site is responsible for stabilization in response to all signals. Indeed, studies using p53 proteins in which all the known and potential N-terminal phosphorylation sites have been mutated to non-phosphorylable residues indicate that phosphorylation is not essential for all forms of DNA damage induced stabilization of p53 (Ashcroft et al. 1999).

p53 itself may not be the only critical target for phosphorylation. Most obviously, Mdm2 has been shown to be phosphorylated (Mayo et al. 1997), for example by ATM, possibly leading to an inhibition of Mdm2's ability to bind p53, function as a ubiquitin ligase or export p53 from the nucleus. Moreover, the hypophosphorylation of Mdm2 central residues is able to inhibit the p53 degradation without influencing its ubiquitin-ligase activity (Blattner et al. 2002) and may involve the Cyclin G1.

In addition to inducing p53 to accumulate, DNA damage is widely believed to activate p53 as a transcription factor through post-translational mechanisms. These phosphorylation events effect the transactivation function of p53. The C-terminal regulatory domain of p53 is a target for phosphorylation by casein kinase 2 (CK2), protein kinase C (PKC) (Herrmann et al. 1991), cdk (Wang and Prives 1995), and p34cdc2 (Milner et al. 1990). p34cdc2 can bind to p53 *in vivo* (Milner et al. 1990) and phosphorylate it *in vitro* (Wang and Prives 1995). Phosphorylation by cell cycle-dependent protein kinases suggests that the activity of p53 is regulated differentially during the cell cycle. Phosphorylation by PKC and casein kinase2 (CK2) *in vitro* stimulate p53 to bind to DNA (Hupp et al. 1993) probably through a conformational change of the protein. Taken together, the data suggest that, *in vivo* the C-terminal domain of p53 may have functions in addition to regulating DNA binding and that constitutive phosphorylation of this domain may be required to elicit such functions.

After the stress signal, de-phosphorylation is necessary to come back to the p53 basal level, but these phosphatases are still poorly studied. Moreover, instead of being phosphorylated, some p53 amino acids have to be de-phosphorylated after stress (Gatti et al. 2000). Hence, inactivity of p53 wt in different tumors may be also explained by the deregulation of one or more phosphatases (Minamoto et al. 2001; Satyamoorthy et al. 2000).

b) Acetylation.

It has been shown that the Histones Acetyl Transferases p300/CBP and PCAF (p300/CBP Associated Factor) are able to acetylate p53 (Goodman and Smolik 2000). After genotoxic stress, PCAF acetylates Lys320, and p300/CBP the Lys372, 373, 381, 382 (Ito et al. 2001; Liu et al. 1999; Sakaguchi et al. 1998) (**Fig.15**). Full length p300/CBP binds to the transactivation domain within the N-terminus of p53, and this interaction is enhanced by the phosphorylation in Ser15, 20, 33 and 37. Acetylation was first supposed to enhance p53 DNA binding, possibly by inhibition of p53's non-sequence-specific DNA-binding activity (Anderson et al. 1997; Gu and Roeder 1997; Lill et al. 1997; Sakaguchi et al. 1998; Liu et al. 1999), but recent studies showed that p53 acetylation was not correlated to its transcriptional activity (Guo et al. 2000; Nakamura et al. 2000; Pearson et al. 2000; Prives and Manley 2001; Rodriguez et al. 2000).

p300/CBP may compete with Mdm2 for its binding site on p53, and for the post translational modifications, as the ubiquitinated Lysines are also the ones acetylated by p300/CBP (Avantaggiati et al. 1997; Grossman et al. 1998), so the phosphorylation of Ser15 and 20 may enhance the p300/CBP binding, more than inhibit the interaction with Mdm2 (Dumaz and Meek 1999).

Moreover, p53 is recruited in the nuclear bodies by PML (ProMyelocytic Leukemia) (Fogal et al. 2000; Guo et al. 2000), hence, co-localizes with p300/CBP in these structures (Ferbeyre et al. 2000; Fogal et al. 2000; Pearson et al. 2000). The p300/CBP HAT activity may enhance p53 transcriptional activity by recruiting the transcriptional machinery directly on the p53 target genes (Espinosa and Emerson 2001) (**Fig.16**).

c) Phosphorylation-independent activation.

Insults such as heat shock, oncogene activation or treatment with actinomycin-D, stabilize p53 without significant phosphorylation of serine 15 (de Stanchina et al. 1998; Nakagawa et al. 1999). Some activating signals have been shown to specifically inhibit the transcription of Mdm2, thereby reducing Mdm2 protein levels and increasing p53 stability (Arriola et al. 1999; Blattner et al. 1999). Cytoplasmic sequestration of p53 would result in a similar inability of p53 to activate Mdm2 expression, with consequent stabilization of the p53 protein. In this case, however, the cytoplasmic p53 protein would also fail to activate transcription of other target genes that are necessary to mediate cell cycle arrest and apoptosis, and is therefore likely to

be defective in activating the full tumor suppressor response (Moll et al. 1996).

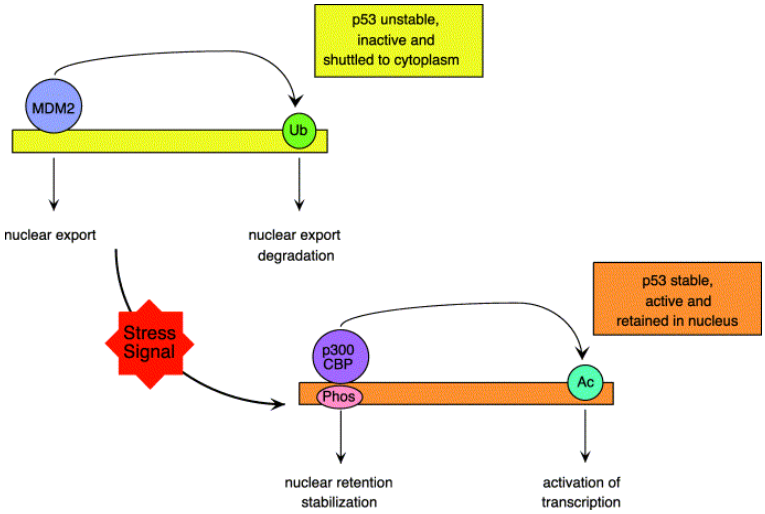


Figure 15. A model for the activation of p53 involving N-terminal phosphorylation.

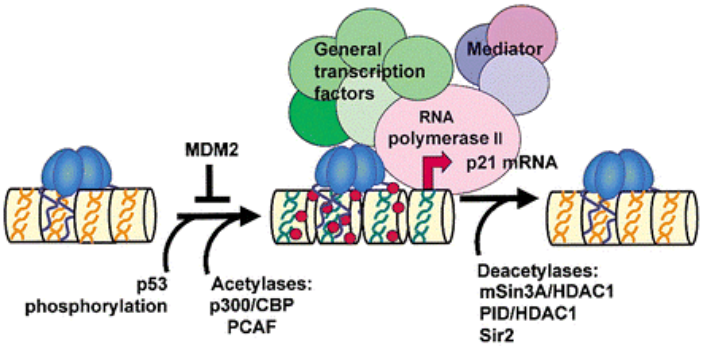


Figure 16. Roles of Acetylation in Transcriptional Regulation by p53.
Association of p53 (blue spheres) with chromatin is not affected by its state of acetylation, but p53-dependent alterations in chromatin structure by histone acetylation and subsequent recruitment of the transcriptional machinery (general transcription factors, mediator and RNA polymerase II) are facilitated by interactions with acetylases, and possibly by acetylation of p53 itself (acetyl groups on p53 and histones are indicated by red circles). Deacetylases serve to repress p53-mediated transcription, likely by returning chromatin to a repressed state. (Prives and Manley 2001).

The DNA-binding activity and conformation of p53 are also regulated by the RedOx state of the protein : oxidation inhibits DNA binding, whereas reduction favors it (Hainaut et al. 1993a,b; Hupp et al. 1993; Rainwater et al. 1995). Several cysteine residues in the core DNA-binding domain have been involved in zinc coordination, and mutational analysis has allowed to identify cysteines at positions 173, 235 and 239 which participate in DNA binding and are also critical for transcriptional activation and suppression of transformation (Rainwater et al. 1995). p53 might be regulated by both the presence of oxygen intermediates and the antioxidant defense mechanism of the cell (Hainaut et al. 1993a,b).

The best understood of the phosphorylation independent stabilization mechanisms at the moment involves activation of expression of a small tumor suppressor protein called $p14^{ARF}$ in humans, $p19^{ARF}$ in the mouse (Kamijo et al. 1998; Pomerantz et al. 1998; Stott et al. 1998). $p14^{ARF}$ binds directly to Mdm2 in a region distinct from the p53 binding domain, and inhibits the degradation of p53 without preventing the binding (**Fig.14**). $p14^{ARF}$ functions both by inhibiting the ubiquitin ligase activity of Mdm2 (Honda and Yasuda 1999) and by sequestering Mdm2 into the nucleolus, thus preventing nuclear export which is necessary for p53 degradation (Weber et al. 1999).

d) Oncogenes activation.

Amongst the stress signals that induce p53, abnormal proliferation driven by oncogene activation has recently emerged as one of the most interesting and important. Cell cycle progression depends to a large extent on the activity of the E2F family of transcription factors. Many of the genes necessary for cell growth are regulated by E2F, and activation of E2F occurs at each cell cycle (Dyson 1998), to allow the cell to enter the S phase. In normal cells E2F activity is tightly regulated by several mechanisms, and loss of this regulation is a common event in cancer cells (Hall and Peters 1996). It appears that abnormal proliferation characteristic of malignant progression is achieved in part by uncontrolled activity of E2F, but in addition to driving proliferation this is also the signal to mechanisms that eliminate cells undergoing such oncogenic changes (Pan et al. 1998; Tsai et al. 1998). One member of the E2F family, E2F1, shows strong apoptotic activities (Nevins 1998) which are in part reflected by the ability of E2F1 to stabilize p53. This stabilization of p53 is achieved through the direct transcriptional activation of $p14^{ARF}$, which inhibits Mdm2 as described above (Bates et al. 1998) (**Fig.14**). Other oncogenes, such as Ras, Myc and

E1A have also been shown to stabilize p53 through $p14^{ARF}$ (de Stanchina et al. 1998; Palmero et al. 1998; Zindy et al. 1998) and the $p14^{ARF}$/Mdm2/p53 pathway represents an important failsafe mechanism to protect the organism from the outgrowth of abnormally proliferating cells.

e) SUMO.

SUMO-1 (Small Ubiquitin-like MOdifier) is an ubiquitin-like protein, first identified as GAP-modifying protein. SUMO is also a binding partner of proteins such as PML and CD95 and is essential for PML localization in the PML oncogene domain. It also covalently modifies IκBα, on the ubiquitin modified lysines, thus inhibiting its degradation.

It has been recently shown that p53 may be also modificated by SUMO-1 on the K386, which is not ubiquitinated nor acetylated (Gostissa et al. 1999; Rodriguez et al. 1999). The sumoylation of p53 seems to activates its transcriptional activity and to modify its cellular localization, even if the p53 mutant K386R is still transcriptionaly active and localized in the nucleus (Kwek et al. 2001). Another recently identified protein, PIAS1 (Protein Inhibitor of Activated STAT1), is involved in p53 sumoylation thanks to its SUMO-ligase activity (Kahyo et al. 2001).

5. p53 activity.

We have seen that after different signals, such as aberrant growth signals, DNA damage or non genotoxic stress such as hypoxia, p53 is stabilized by post-translational modifications. The p53 protein functions as a tetrameric transcription factor and its activation leads either to cell cycle arrest to contribute directly or indirectly to DNA repair, or to programmed cell death (depending on the cellular context, the extent of damage and other unknown parameters) (**Fig17**). Precise mapping of the binding sequences revealed a consensus binding site with a striking internal symmetry consisting of two copies of the 10 base-pair motif 5'-PuPuPu-C(A/T)(T/A)GPyPyPy-3' separated by 0-13 nucleotides.

The number of genes transactivated by p53 might be as many as several hundred, at least when p53 is artificially overexpressed (Zhao R et al. 2000, Yu et al. 1999, Kostic et al. 2000, Kannan et al. 2001a,b). By DNA-chip analysis, numerous genes have been found to be activated by p53 : genes responsible for cellular adhesion, signal transduction, transcription, cell growth, cytosqueletal formation, extra cellular matrix, growth factors.

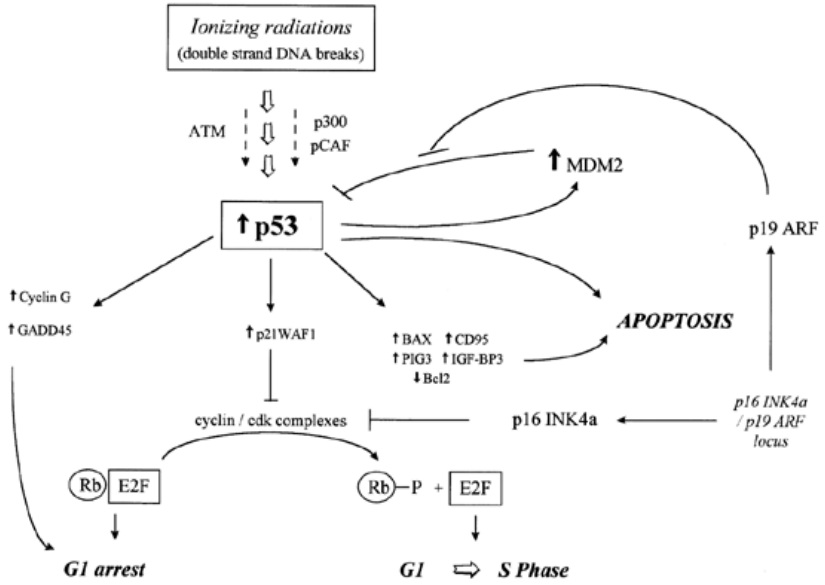


Figure 17. Schematic representation of the events occurring after ionizing irradiation of cells with a wt p53 gene.
The outcome of the cellular response is either cell growth arrest or apoptosis, depending on several determinants, including the cell type, the extent of DNA damage, the level of p53 and the genetic background. Ionizing irradiation produces DNA strand breaks that result in accumulation and activation of p53. Optimal induction of p53 after irradiation appears to require a wild type ATM product. Activation of p53 results in the transcriptional transactivation of many genes (GADD45, mdm2, p21 WAF1, BAX, Cyclin G, IGF BP3, PIG3) and in the down-regulation of several genes including BCL2. In cells in which the outcome is G1 arrest, an increase in p21 WAF1 protein contributes to the arrest of cells in G1, through inhibition of cyclin-cdk complexes, with a subsequent accumulation of unphosphorylated form of RB which arrests cells in G1 by preventing the release of E2F from its complex with RB. E2F is required for the G1-to-S transition. In certain cells, the outcome is apoptosis. It is likely to be mediated by increased levels of Fas/APO1/CD95, BAX, IGF BP3 and PIG3. In addition the products of INK4a/ARF locus, namely p16INK4a and p19 ARF are involved at the junction of p53 and RB pathways. p16INK4a is an inhibitor of cyclin-dependent kinase that functions upstream of RB. p19 ARF blocks MDM2 inhibition of p53 activity. (May 1999)

Nevertheless, all these studies have been realized on tumoral cells and only a few of these genes have been fully validated in normal cells or tissues. In fact, it seems that the p53-induced response is highly dependent on the p53 level in the cell, the inducing signal and, above all, on the genetic status of the cell. A tumoral cell may lack some p53-dependent or –independent regulation pathways. Additional data showed that p53 may down-regulate the expression from a number of other promoters (Ginsberg et al., 1991; Lechner et al., 1992; Ko and Prives, 1996). Finally, recent research suggests that p53 may participate in the repair machinery (Smith et al. 1994; Wang XW et al. 1995a; Ford and Hanawalt 1995; Li et al. 1996).

a) Cell cycle arrest and DNA repair.

i *G1-S and G2-M transitions.*

Once activated, p53 is able to block cell cycle in G1-S or in G2-M. This arrest is principally due to the activation of two genes : $p21^{WAF1/CIP-1}$ for the G1-S arrest, and 14-3-3σ for the G2-M one (El-Deiry et al. 1993; Hermeking et al. 1997; Kastan et al. 1992). Other genes involved in cell cycle arrest, such as GADD45, are also transactivated by p53 (See **Fig.7**).

The $p21^{WAF1}$ protein is a CDKI (Cyclin Dependent Kinase Inhibitor) and was identified as a potent inhibitor of several CDKs, including cyclinD-CDK4/6, cyclin E-CDK2 and cyclin A-CDK2. One potential downstream target of $p21^{WAF}$ inhibitory activity in G1 is the cell cycle-dependent phosphorylation of the retinoblastoma (RB) protein. RB is in a hypophosphorylated form during G1. In this state it binds to and sequesters the S phase promoting E2F family of transcription factors (Sherr 1998; Giaccia and Kastan 1998). Advancement through the cell cycle is thought to be mediated by sequential phosphorylation of RB by G1 cyclin-dependent kinases, resulting in the release of active E2F, which then leads to the transcriptional activation of genes required for S phase progression (Sherr 1998; Giaccia and Kastan 1998). In this pathway $p21^{WAF1}$ leads to the inhibition of cyclin D-CDK4/6 complexes and subsequent accumulation of the unphosphorylated form of RB, which arrests cell in G1. This G1 arrest is supposed to prevent the replication of damaged DNA and to allow DNA repair before entry into S phase.

Moreover, $p21^{WAF1}$ has also been implicated in regulating DNA replication. $p21^{WAF1}$ directly interacts with PCNA (Proliferating Cell Nuclear Antigen). p53 inhibits PCNA-dependent DNA replication (but not DNA repair) in *in vitro* systems (Flores-Rozas et al. 1994; Li R et al. 1994; Waga

et al. 1994), decreasing the DNA-polymerase activity. p53 protein has also been involved in directly inhibiting DNA replication through its association with the single-stranded DNA-binding protein complex RPA (Dutta et al. 1993) that is required for DNA unwinding during replication (See **Fig.6**).

The 14-3-3σ protein is involved in the G2-M transition by sequestering the phosphorylated form of cdc25c, thus blocking the activation of the complex Cyclin B-Cdc2 (Hermeking et al. 1997). Moreover, 14-3-3σ is directly involved in DNA repair (Chan TA et al. 2000).

GADD45 is able to block the cell cycle either in G1, interacting with PCNA (Smith et al. 1994; Wang et al. 1999), or in G2, interacting with the complex CyclinB-Cdc2 (Zhan et al. 1999).

ii *DNA repair.*

p53 induces the transcription of genes such as xpc (Xeroderma Pigmentosium group C), hHr23b, Ddb1 and Ddb2 (Sugasawa et al. 1997, 1998; Adimoolam and Ford 2002; Hwang et al. 1999; Tang et al. 2000). All these proteins are involved in the NER (Nucleotide Excision Repair), and especially, in the Global Genomic Repair (See **Fig 6**). GADD45 seems to be also involved in GGR, but its role is poorly understood (Smith et al. 2000).

Moreover, it has been shown that p53 binds to and modulates the repair activity of the nucleotide excision repair factors XPB and XPD (Wang XW et al. 1994, 1995a). More recently it has been demonstrated that p53 interacts with human Rad51 protein, a proposed member of the mammalian recombination machinery (Buchhop et al. 1997). Finally, p53 has been shown to bind single-stranded DNA ends (Bakalkin et al. 1994) and to possess strong DNA-DNA and RNA-RNA strand annealing activity (Bakalkin et al. 1994; Oberosler et al. 1993; Brain and Jenkins 1994).

b) Apoptosis induction.

Apoptosis induction is essential in the p53 tumor suppressor activity, as it allows to eliminate potentially tumoral cells, with damaged DNA, aberrant growth factors activation or deregulated RedOx status. p53 is able to induce cell death either by activating the transcription of target genes, either by mechanisms transcriptionaly independent, depending on the genetic status of the cell (Haupt et al. 1995; Venot et al. 1998).

i p53 target genes.

p53 activates the transcription of numerous genes involved in apoptosis induction. The proteins encoded are localized in different cellular compartments (cytosol, mitochondria or plasmic membrane) and play different roles. Most of them present a p53 responsive element in the promoter or in an intron, but recent DNA chips analysis allowed to characterize new genes transactivated after p53 induction (Kannan et al. 2001b). The major p53-induced proteins are presented in the following **Table 1**, according to their cellular localization.

Moreover, p53 is able to repress transcription of different genes, and this function seems to be important for apoptosis induction, even if the mechanism is still poorly understood (Murphy et al. 1996). Until now, all the genes found to be repressed by p53 have anti-apoptotic functions and are not involved in cell cycle. The most important are : Bcl-2, which blocks CytochromeC release (Miyashita et al. 1994, Newmeyer et al. 2000); WT1 (Wilm's tumor suppressor gene), which binds to p53 and inhibits p53-dependent apoptosis without affecting p53-dependent growth arrest (Maheswaran et al. 1995); β-catenine (Sadot et al. 2001); Map4 (Microtubule Associated Protein 4) and also c-fos, c-jun, c-myc, b-actin, hsc-70, IL-6 (Ginsberg et al. 1991; Lechner et al. 1992; Ko and Prives 1996).

Gene expression is regulated by the acetylation level of Histones : the more they are de-acetylated, the more DNA is compact and the access to the gene is difficult for the RNA polymerase. Hence, the interaction of p53 with the complex mSin3a/HDAC1, which may involve the p53 Proline-Rich region, may be important for its capability to repress genes expression. Another explanation could be the association between p53 and the cellular TBP (TATA-binding protein) which is a component of the TFIID complex (Seto et al. 1992; Ragimov et al. 1993; Truant et al. 1993).

ii p53-dependent apoptosis, transcriptionaly independent.

Different studies showed a pro-apoptotic role of p53 without transactivation of the target genes, or in presence of p53 mutants del(1-124) or Q22/S23, in different cellular models (Caelles et al. 1994; Kokontis et al. 2001; Chen XB et al. 1996; Haupt et al. 1997b; Haupt et al. 1995). In mouse fibroblasts without nuclei, p53 is able to induce the caspase cascade, which is blocked by a p53 depletion and suggests a protein/protein interaction maybe with caspase 8 (Ding et al. 2000).

Mitochondrial proteins	**Bax (Bcl-2-Associated protein)** Contains a BH3 (Bcl-2 Homolog) domain,which allow Cytochrome C release. The oncoprtein c-Myc cooperates with Bax to induce apoptosis. (Miyashita & Reed 1995; Rosse et al. 1998; Juin et al. 2002) **NOXA** Interacts with the anti-apoptotic members of Bcl-2 family (Bcl-2, BclXl, Mcll) thanks to its BH3 domain. (Oda et al. 2000a) **PUMA (p53 Upregulated Modulator of Apoptosis)** Contains a BH3 domain and induces rapid Cytochrome C release. (Nakano & Vousden 2001; Yu et al. 2001) **p53AIP (p53 regulated Apoptosis Inducing Protein1)** Its induction depends on p53 phosphorylation on Ser46 by p53DINP1 (p53 Dependent Damage Inducible Nuclear protein 1) also activated by p53. WIP1 is able to decrease the Ser46 phosphorylation after stress. (Oda et al. 2000b; Okamura et al. 2001; Takekawa et al. 2000)
Cytoplasmic proteins	**PIG3 (p53 Induced Gene 3)** Involved in free radicals generation, which alter the mitochondrial membrane and induce CytochromeC release. The p53 responsive element is different from the one previously described. (Polvak et al. 1997; Contente et al. 2002) **PIDD (p53 Induced protein with Death Domain)** Contains a Death Domain homolog to the death receptors. It may interact with another Death Domain protein, RAIDD, and activate Caspase2 by a still unknown mechanism. (Lin et al. 2000) **APAF1 (Adaptor molecule Apoptosis protease Activating Factor 1)** Oligomerizes after Cytochrome C binding, and in presence of ATP or dATP is able to activate Caspase9. (Cain et al. 2000; Fortin et al. 2001)
Membrane-Bound proteins	**Fas/CD95/APO1** Member of the TNF Receptor Family (Tumor Necrosis Factor), is induced by DNA damage p53-dependent and other stimuli, is activated by Fas-ligand and transduces the signal via its Death Domain. Its activation by p53 seems to be tissue specific. (Muller et al. 1998; Owenschaub et al. 1995; Lin et al. 2002) **Killer/DR5 (Death Receptor 5)** Another TNF-R, activated by TRAIL. (Wu et al. 1997; Ashkenazi and Dixit 1998) **PERP (p53 apoptosis Effector Related to Pmp22)** Its over-expression induces apoptosis, activated by Doxorubicin treatment. (Attardi et al. 2000) **Scotin** Localized in plasmic membrane and Endoplasmic Reticulum, induces apoptosis p53- and caspase-dependent (Bourdon et al. 2002)

Table 1. p53-induced genes, presented according to their cellular localization.

Moreover, it has been recently shown that a part of endogenous p53 localizes on the mitochondria after stress (Marchenko et al. 2000; Mihara et al. 2003), and may interact with the anti-apoptotic Bcl-2 family proteins. Finally, it has been proposed that p53 may re-localize the Fas/CD95 receptor from the reticulum and Golgi towards the plasmic membrane.

c) The choice between cell cycle arrest and apoptosis.

One of the main uncertainties in the p53 pathway concerns whether growth arrest or apoptosis occurs. The apoptosis pathway is more easily triggered in transformed cells than in normal cells, indicating that only studies of normal cells or normal tissues will be able to define the mechanism that decides cell fate after p53 induction. Nevertheless, this choice involves both p53 internal regions and some p53 physical partners (**Fig.18**).

p53 presents two transactivation domains AD1 (1-42) and AD2 (43-63). Different studies showed that mutants in the AD1 domain are still able to transactivate pro-apoptotic genes, such as Bax, but not $p21^{WAF}$ (Lin et al. 1995; Venot et al. 1999; Candau et al. 1997). Moreover, a deletion in the proline-rich region (62-91) totally abolish the apoptosis induction but not the cell-cycle arrest (Sakamuro et al. 1997; Venot et al. 1998; Walker and Levine 1996; Zhu et al. 1999), probably by a decrease of interaction with DNA which induces a lack of transactivation or repression of its target genes.

Recently, two new p53 binding proteins have been identified : ASPP1 and 2 (Apoptosis Stimulating Protein of p53). ASPP2 is the full length form of the well known p53BP2 (p53 Binding Protein2), which interacts with the p53 core domain thanks to its Ankyrin motives (Iwabuchi et al. 1994; Gorina and Pavletich 1996; Samuels-Lev et al. 2001). The ASPPs proteins are able to increase the p53 binding on pro-apoptotic genes promoters, without changing its binding on cell-cycle genes promoters. It seems that the ASPP N-terminal domain is important in this regulation and that the interaction with p53 is enhanced by UV treatment (Samuels-Lev et al. 2001).

Finally, the localization of p53 in the nuclear bodies, thanks to PML, allows the action of p300/CBP and PCAF, activated by co-factors such as JMY and PAF400 (Shikama et al. 1999; Vassilev et al. 1998) after DNA damage, and consequently induces the p53-dependent transcription of pro-apoptotic genes (**Fig.19**).

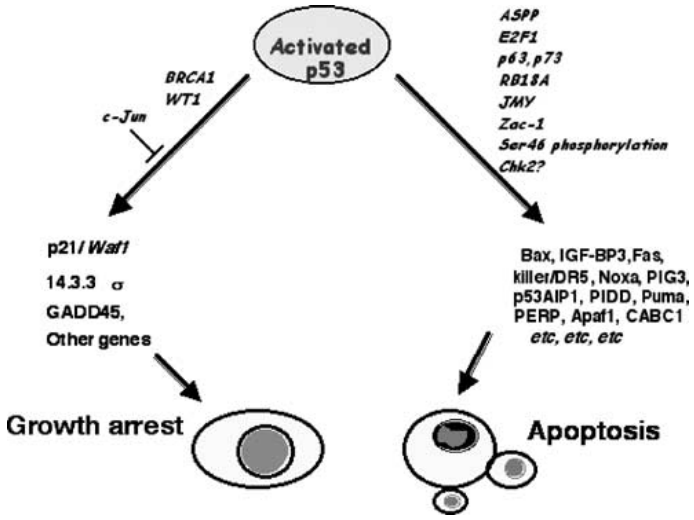


Figure 18. Target genes subject to positive transcriptional regulation by p53 mediate its different biological outcomes. (Oren 2003)

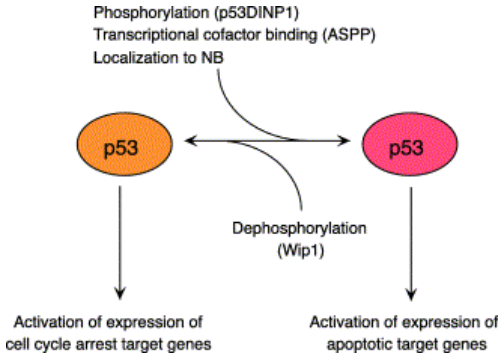


Figure 19. Regulation of the choice of response to p53.
Activation of p53 leads to the induction of genes that mediate cell cycle arrest. However, additional signals that can lead to the phosphorylation of p53, the availability of certain transcriptional cofactors such as ASPP or JMY, and localization of p53 to NBs can modify the response to allow activation of apoptotic target genes and induction of programmed cell death. These responses are controlled to some extent by feedback loops, since both phosphorylation and dephosphorylation can be regulated by proteins (p53DINP1 and WIP1) that are products of p53-inducible genes. (Vousden 2001)

B. p73 functions.

1. p73 : homologies and differences with p53.

a) p73 different isoforms : structure and function.

i *A highly-conserved family protein.*

The three members of the p53 family (p63, p73 and p53) share significant homology both at the genomic and at the protein level. p63 and p73 contain the characteristic features of the p53 protein : an acidic, amino-terminal transactivation (TA) domain, a central core DNA-binding (DBD) and a carboxy-terminal oligomerization domain (Kaghad et al. 1997). p73 shares 62% identity with the DNA-binding region of p53, 22% identity with the tetramerization domain, and 18% with the transactivation domain (**Fig.21**). The highest level of homology is reached in the DBD, suggesting that they can bind to the same DNA sequence and transactivate the same promoters. Strikingly, even the major hot spots for mutation of p53 in human cancers are highly conserved among the p53 homologue (Kaghad et al. 1997). Indeed, in experimental systems, p73 showed many p53-like properties : it could bind to p53 DNA target sites, transactivate p53-responsive genes and induce cell cycle arrest or apoptosis (Jost et al. 1997; Zhu et al. 1998; Ishida et al. 2000). Since the discovery of p73 and p63, a large body of evidence has demonstrated their involvement in DNA damage as well as in development (**Fig.20**).

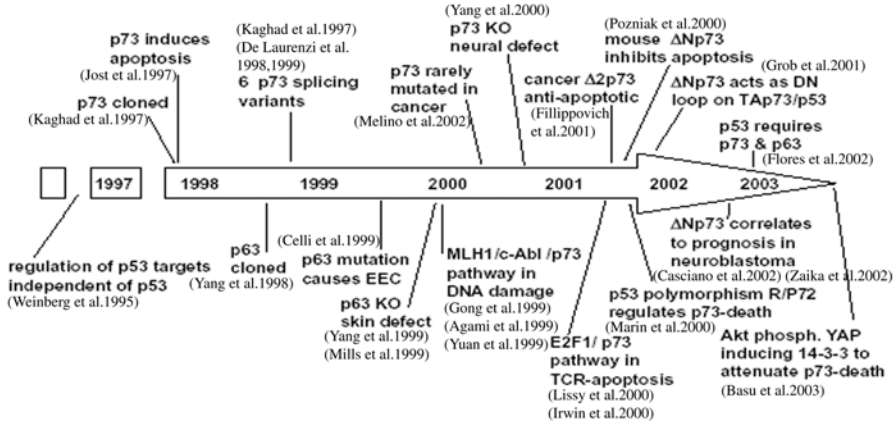
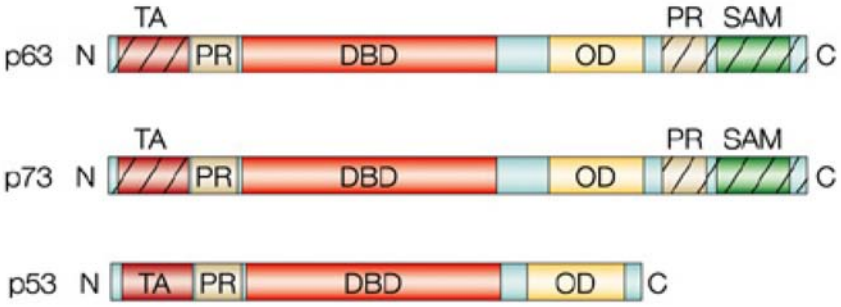


Figure 20. Timeline of the p53 family. (Maisse et al. 2003)



p73 vs. p63	37% (25%)	91% (86%)	74% (53%)	83% (53%)
p73 vs. p53	30% (18%)	79% (62%)	33% (22%)	-
p63 vs p53	26% (6%)	80% (58%)	31% (19%)	-

Figure 21. The p53 family.
The overall domain structure of p53, p63 and p73 is conserved. In contrast to p53, p63 and p73 have many different isoforms with distinct amino (N) and carboxyl (C) termini. Full length isoforms contain the transactivation domain, so are designated TA; amino-terminal-deleted isoforms are designated ΔN. Dashed lines indicate different isoforms in N and C-terminal domain. The percentage of homology and identity is indicated below. (Adapted from Melino et al.2002)

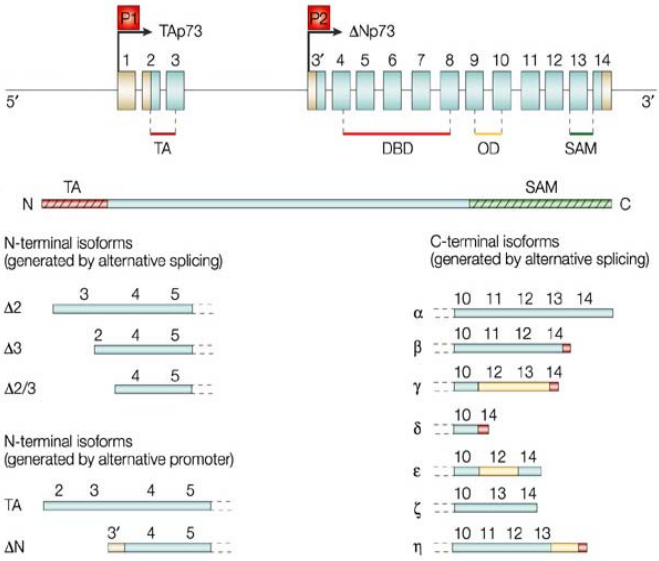


Figure 22. p73 isoforms. (Melino et al. 2002)

ii *The C-terminal domain.*

Conversely to p53, p73 and p63 contain additional C-terminal extensions. In both proteins, these extensions show alternative splicing, which results in at least six C-terminal variants for p73 (p73 α–ϕ) and three for p63 (α–γ) (Kaghad et al. 1997; De Laurenzi et al. 1998; Irwin and Kaelin 2001) (**Fig.22**). These isoforms have different transcriptional and biological properties, and their expression patterns change among normal tissues (Irwin and Kaelin 2001). For example, p73β transactivates many p53-responsive promoters, and p73α does so at a lesser extent (Jost et al. 1997; Zhu et al. 1998; Di Como et al. 1999). Nonetheless, the role of the several isoforms in cellular function is far from being fully understood, and it has been shown that their differential regulatory roles are highly cell-context dependent (Freebern et al. 2003).

The α variants of p73 and p63 have close to their C terminus a SAM (Sterile Alpha Motif) domain, which is thought to be responsible for regulating p53-like functions (Bork and Koonin 1998). SAM domains are protein modules of approximately 65- to 70-amino acids found in diverse proteins whose functions range from signal transduction to transcriptional repression (Bork and Koonin 1998). Interestingly enough, it has been reported that the α isoform of p73 (and also that of p63, as we will see below) has its p53-like function dramatically reduced in comparison with other non SAM-containing isoforms, suggesting that SAM domain, or a post-SAM domain, could be responsible for those functional differences (Thanos and Bowie 1999; Bork and Koonin 1998).

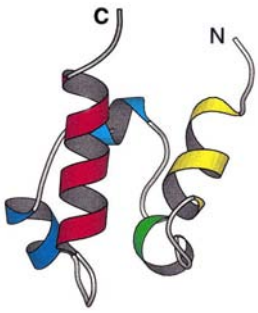


Figure 23. A ribbon diagram representing the backbone of the SAM domain of p73. (Arrowsmith 1999)

The structure of the SAM domain of p73, SAMp73, has been resolved by NMR (Chi et al. 1999) and X-Ray crystallography (Wang W. et al. 2000; Wang W. et al. 2001) (**Fig.23**). The domain (residues 487-554 of the full p73α protein) contains a single tryptophan residue, which could be used as a spectroscopic probe to monitor the protein conformational changes. The structure of the domain reveals a small five-helix bundle composed of four α-helices (residues 491-499 (helix 1), 506-511 (helix 2), 525-531 (helix 4) and 538-550 (helix 5)), and a small 3_{10}-helix (residues 517-520, (helix 3)). The SAMp73 has a structural similarity with two ephrin receptors tyrosine kinases (Chi et al. 1999), and the spatial arrangement of the bundle is similar to that of SAM domains found in other proteins (Thanos and Bowie 1999). SAM domains are putatively considered to be responsible for regulating protein functions *via* self-association or by association with other domains (Schultz et al. 1997), but the exact function of SAMp73 is not known. The crystal structure of SAMp73 reveals a dimeric organization (Wang et al. 2000), but the NMR structure is monomeric (Chi et al. 1999), suggesting that dimer formation in the crystal is an effect of crystal packing rather than a real physiological state; furthermore, equilibrium sedimentation experiments have shown that SAMp73 is monomeric under a wide range of experimental conditions (Chi et al. 1999; Barrera et al. 2002).

In fact, SAM domains often mediate homodimerization, but this is not the case for the SAM domain of p73. Nevertheless, p73 can homodimerize and heterodimerize (both with other splice variants, and, to a much lesser extent, with wild-type p53 and some of its mutant forms) through the oligomerization domain.

iii *The ΔNp73 isoform.*

Another difference with p53 is the N-terminal truncated p73 isoform, which has been first described in the mouse. In 2000, Pozniak et al. demonstrated that the apoptotic function of p53 in mouse neurons is modulated by p73 (Pozniak et al., 2000). The predominant isoforms of p73 *in vivo* are truncated proteins that lack the N-terminal transactivation domain: ΔNp73 (Pozniak et al., 2000; Yang et al., 2000) (**Fig.24**). Moreover, overexpression of ΔNp73 isoforms inhibits sympathetic neuron apoptosis caused by NGF withdrawal or p53 overexpression, and developmental death of sympathetic neurons is enhanced in p73 -/- animals (Pozniak et al. 2000). Because the only detectable isoform of p73 in mouse sympathetic neurons is ΔNp73β, a molecule whose levels are up-regulated by NGF (Pozniak et al., 2000), these findings indicated that ΔNp73 functions in the developing PNS

as an essential anti-apoptotic molecule, potentially by antagonizing the pro-apoptotic functions of p53. Finally, observation of p73 -/- CNS structures of 14 days mice led to conclude that ΔNp73 isoforms function as essential pro-survival molecules in both the CNS and PNS and are important not only during the period of developmental death but also for the maintenance of at least some populations of adult neurons.

The amino-terminally truncated ΔNp73 isoforms are generated by using an alternative promoter (P2) that is located in intron 3 (Grob et al. 2001) (**Fig.22**). They therefore lack the TA domain, which is encoded by the first three exons of TP73, and contain an additional exon (exon 3'). p73 isoforms that lack the amino-terminal TA domain can also arise from alternative splicing of transcripts that originate from the first exons (Ishimoto et al. 2002; Fillipovitch et al. 2001). Δ2p73 and Δ3p73 are splicing isoforms that lack exons 2 and 3, respectively; these forms are associated to tumoral cells.

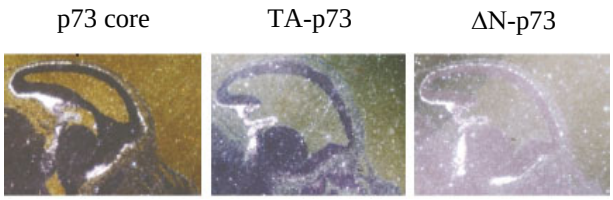


Figure 24. ΔN isotype is predominantly expressed.
In situ hybridization on embryonic day 12 mouse brain sections using various p73 probes to distinguish between TA and ΔN expression. The patterns of expression with the core and ΔN probes are nearly identical, whereas TA shows signal in only a small percentage of p73-positive cells. (Yang et al. 2000)

Intriguingly, whereas in mouse brain tissues ΔNp73 seems to be the most highly expressed isoform (Yang et al. 2000), the TA forms are most abundant in human fetal and adult tissues (Ishimoto et al. 2002; Grob et al. 2001). The possibility that ΔNp73 has different roles in different embryonic and adult tissues still awaits experimental support. Whereas TAp73 isoforms function as transcription factors that can induce irreversible cell-cycle arrest and apoptosis, the ΔNp73 isoforms that lack the TA domain are incapable of inducing gene expression directly and do not induce growth arrest or cell death.

However, the ΔNp73 forms have a very important regulatory role, as they exert a dominant-negative effect on p53 and TAp73 by blocking their transactivation activity, and hence their ability to induce apoptosis (Ishimoto et al. 2002; Grob et al. 2001). This inhibitory function is exerted either at the oligomerization level (for TAp73), or by competing for binding to the same DNA target sequence (for p53) : although the ΔNp73 forms can interact with TAp73, they seem to have lost the ability to interact with wild-type p53 (Ishimoto et al. 2002; Grob et al. 2001; Stiewe 2002) (**Fig.25**).

ΔNp73 isoforms can therefore control the activity of both TAp73 and p53, which makes the fact that the ΔNp73 promoter (P2) contains a very efficient p53/TAp73 responsive element particularly interesting (Nakagawa et al. 2002; Grob et al. 2001). p53 and TAp73 can induce expression of the ΔNp73 isoform, which creates a dominant-negative feedback loop that regulates the function of both p53 and TAp73, and can fine-tune the function of p53 in a manner that is similar to the Mdm2 loop (**Fig25**). The ability of p53 and TAp73 to control the expression of the ΔNp73 isoform through this p53-responsive element has also been confirmed by other reports (Vossio et al. 2002; Kartasheva et al. 2002).

Both Mdm2 and ΔNp73 are direct transcriptional targets of p53, and they are able to inhibit p53 function by inducing its degradation (in the case of Mdm2) or competing for its target DNA-binding sites (in the case of ΔNp73). Perturbations of these regulatory loops in cancer cells (Stiewe et al. 2002) or in virally infected cells (Allart et al. 2002), resulting in excess or persistent expression of Mdm2 or the ΔNp73 isoform, might result in an inability to activate p53 or TAp73. Consequently, loss of these regulatory pathways would be predicted to allow inappropriate p53 or TAp73 activity and inhibition of cell growth, contributing to cancer development. Indeed, deletion of Mdm2 results in early embryonic lethality that is directly dependent on p53 activity (Jones et al. 1995; Parant et al. 2001). Unfortunately, the knockout TP73 mouse is not fully informative in this respect, as expression of both the TAp73 and ΔNp73 isoforms is lost. The *in vivo* evaluation of the TAp73 and ΔNp73 isoforms awaits the creation of selective knockout mice.

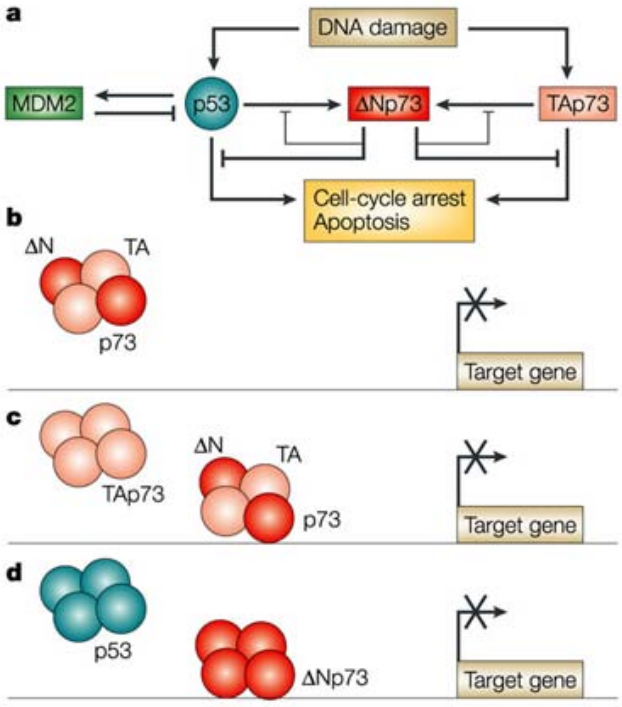


Figure 25. Regulation of ΔNp73 by p53 and TAp73.
The isoform ΔNp73 is regulated by its own promoter,which contains a highly active p53/p73-responsive element. **a)** Both p53 and TAp73 are activated in response to DNA damage and transcriptionally transactivate ΔNp73; this, in turn, downregulates its own promoter to fine-control the steady-state protein levels. Conversely, ΔNp73 is able to functionally inhibit cell-cycle arrest and apoptosis that is induced by either TAp73 or p53. The ΔNp73 regulatory loop is reminiscent of the MDM2 loop; whereas the latter regulates p53 degradation, the former exerts a functional inactivation, allowing tight synergistic control of p53/TAp73 function. The inhibition of apoptosis that is exerted by ΔNp73 is due to **b)** sequestration of TAp73, **c)** formation of less-active TAp73–ΔNp73 heterodimers or **d**) competition with p53 for the same p53 promoter-targeting sequences. ΔNp73 therefore forms a dominant-negative loop in the regulation of the function of both TAp73 and p53. (Melino et al. 2002)

b) p53 family evolution : from p63 to p53, *via* p73.

An interesting debate is simmering on the evolutionary relationships among p53 family members, the outcome of which will probably affect our thinking on their mechanisms of action, regulation and functional

interactions. The issue is : which member of the vertebrate p53 family came first? The mouse knockout phenotypes might argue that TP63 or TP73 was the original gene : as we'll see more in detail, the developmental and physiological consequences of their disruption are severe and affect basic physiological processes, whereas TP53-/- mice, apart from their susceptibility to tumors, appear normal.

Alternatively, protection against genomic damage, rather than tumor suppression *per se*, may be a more ancient concern of an organism, and the antecedent gene might behave more like TP53 as we know it in vertebrates. So far, comparative genomics has not provided any conclusive answers : the *Drosophila melanogaster* genome encodes a single p53-like protein (Dmp53) for which no genetic data are yet available. However, Dmp53 can transactivate genes and is pro-apoptotic. Moreover, dominant-negative experiments and sequence homology have been used to argue that Dmp53 and human p53 lie on a direct evolutionary line (Ollman et al. 2000; Brodsky et al. 2000; Jin et al. 2000). This would argue that p63 and p73 arose by gene duplication from the original "tumor suppressor" p53 and later assumed their present functions in epithelial stem-cell maintenance, secretion and neurogenesis. We cannot be more definitive here because p63 and p73 can transactivate p53 target genes, and several p63 and p73 isotypes induce apoptosis in mammalian cells (Kaghad et al. 1997; Yang et al. 1998; Jost et al. 1997).

Strictly at the level of amino-acid sequence, Dmp53 is more closely related to the p63 and p73 homologues in mollusks (Ishioka et al. 1995) and human p63 than it is to human p53. Obviously we are dealing with incomplete information here : other than Dmp53, the only invertebrate p53 homologues characterized so far are from squid, whose p53 homologue is most related to p73, and clam, which has one most similar to human p63. Bona fide p53 orthologues are not apparent until the vertebrate branch of the evolutionary tree, whereas zebrafish, *Xenopus* and birds all have p63/p73 orthologues. Human p63 is much more similar to p73 than either is to p53. At first glance, then, it can be argued that p63 was the original gene that evolved in invertebrates, and p73 arose by a gene-duplication event before vertebrate evolution. As p53 is closer in sequence to p73 than to p63, we could surmise a further gene-duplication event during chordate evolution, which gave rise to the TP53 gene. Curiously, the p63 protein shows almost no deviation at the aminoacid level between mouse and humans (99% identity), p73 shows more (96% identity), whereas p53 is surprisingly divergent (86% identity).Whether the conservation of p63 and p73, in

contrast to the TP53 gene, reflects constraints imposed by co-evolving interactions, dispensability or "evolvability" of p53 function is unclear at present (Yang et al. 2002; Melino et al. 2002; **Fig.26**).

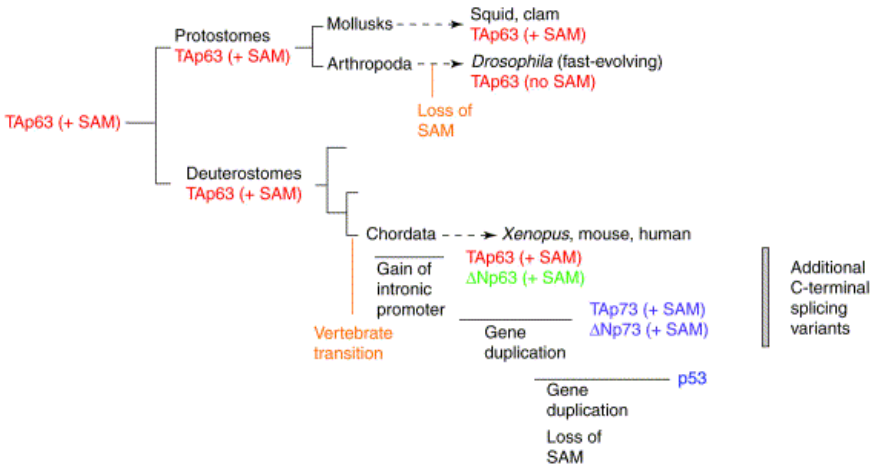


Figure 26. Evolution of the p53 family members.
Major branches of metazoan evolution are shown together with known p53 family members. That the primordial p53 family member is most similar to a TA-p63 protein bearing a C-terminal SAM domain is suggested by the presence of TA-p63(SAM) molecules on both side of the split between deuterostomes and protostomes. On the protostome side (represented by mollusks and arthropods such as Drosophila), TA-p63 with or without SAM domains is the only p53-like molecule reported. In contrast, the deuterostome branch, and in particular the vertebrates, show TA-p63, ΔNp63, TA-p73, ΔN73 and p53, suggesting a co-evolution of ΔN isoforms with p73 and p53. (Yang et al. 2002)

2. p73 and the tumoral process.

When TP73 was identified on chromosome 1p36 and shown to be the first p53 homologue, the obvious question was whether TP73 was the long sought-after tumor suppressor at 1p36, a locus that is commonly deleted in various tumours. An extensive investigation into the p73 status of primary tumours (particularly those with 1p36 deletions, as is the case with neuroblastoma) was initiated by several groups and over 1500 tumours have

been screened. TP73 mutations are quite rare : present in fewer than 0.5% of tumours, compared with over 50% that had TP53 mutations. There is, however, a significant incidence of LOH (Loss Of Heterozygosity) in several cancer types, although there is no clear evidence that the remaining allele is inactivated in these cases. So, the TP73 story is more complex than for classical tumor-suppressor genes, such as its relative TP53, and other possibilities may be considered, such as the fact that 1p36 may harbor a tumor-related gene other than TP73.

The p73-null mice confirm these clinical observation : there have been described severe developmental abnormalities, but no increase in the tumor formation rate is detected, as it happened in p53-knockout mice (Yang et al. 2000; Melino et al. 2002).

a) Two genes in one.

In fact, the intricacies of p73 regulation are only now beginning to be understood. One of the more obvious complications in assessing the role of p73 in tumor development is the fact that the TP73 locus encodes both a tumor suppressor (TAp73) and a putative oncogene (ΔNp73). Interpretation of the observations that TP73 expression is increased in many tumours (Ikawa et al. 1999), and that mice that are deficient for TP73 do not seem to be tumor prone (Yang et al. 2000), is hampered by the lack of discrimination between expression of the TAp73 and ΔNp73 isoforms. Overexpression of ΔNp73 isoforms in cancers might reflect inactivation of bona fide tumor suppressor activities of the TAp73 isoforms. An assessment of tumor incidence in mice that are selectively deleted for the TAp73 or ΔNp73 isoforms might reveal their function in tumor development.

Furthermore, as the same exons of p73 encode the DNA-binding domains of both TAp73 and ΔNp73, mutations within this region (which is where most tumor-associated mutations in TP53 are found) would inactivate both the growth-inhibitory activity of the TAp73 proteins and the ability of ΔNp73 to inhibit p53 function. Such mutations would, therefore, be predicted to abrogate both tumor-suppressor and oncogene activities of the TP73 locus, and might explain the absence of such mutations in cancers. Further work is, therefore, needed to assess the relative role of TAp73 and ΔNp73 in tumor development.

b) The p73 N-terminal variants and the tumoral process.

Recent studies that discriminate between the TAp73 and the ΔNp73 isoforms indicate that expression of the latter might be an important determinant in cancer development. Interestingly, several amino-terminally deleted versions of TP73 are expressed in cancer cells, including ΔNp73, Δ2p73 and Δ3p73 (Fillipovitch et al. 2001). All of these p73 variants have anti-apoptotic features, which could be relevant for the progression of the tumor; indeed, the Δ2p73 isoform is not expressed in normal tissues, but is overexpressed in breast cancer cell lines (Fillipovitch et al. 2001), ovarian cancer (Ng S W et al. 2000), vulval cancer (O'Nions et al. 2001) and neuroblastic tumours (Douc-Rasy et al. 2002).

So far, however, there is only one study on the prognostic value of ΔNp73-isoform expression (Casciano et al. 2002a). A study of 52 patients who are affected by neuroblastoma showed that expression of the ΔNp73 isoform is a strong adverse prognostic marker, independent of stage and N-Myc amplification. ΔNp73-negative patients had an overall survival rate of ~80%, with 65% progression free survival at 5 years, compared with ΔNp73-positive patients, none of whom survived. The potential oncogenic function of ΔNp73 is also indicated by the observation that overexpression of the ΔNp73 isoform results in malignant transformation of NIH-3T3 fibroblasts and tumor growth in nude mice (Stiewe et al. 2002).

Taken together, these data indicate that the functions of ΔNp73, such as the inhibition of p53- and TAp73-induced apoptosis, could be important for the development of the tumor. In such a scenario, it is not necessary to mutate and inactivate the gene, but merely to modulate the TA:ΔN ratio by regulating their respective promoters, for example, by methylation (Casciano et al. 2002b).

c) The p53 Arg/Pro polymorphism.

More than 50% of all human cancers have mutations in p53 that inhibit its function. Whereas p53 has a moderate degree of interaction with p73 (Kaghad et al. 1997, DeLaurenzi et al. 1998), some, but not all, tumor-derived p53 mutants (which have lost p53 tumor-suppressor functions) have a significant ability to bind and interact with TAp73α (Di Como et al. 1999; Gaiddon et al. 2001; Strano et al. 2000). This interaction occurs through the p53 core domain, rather than its tetramerization domain, and the acquisition of p73 binding activity seems to contribute to the oncogenic activity of these

p53 mutants by allowing them to sequester and inactivate p73. In a further refinement of this observation, Marin et al. (2000) reported that the interaction between mutant p53 and TAp73 depends on the TP53 polymorphism at codon 72. p53 proteins that carry the inactivating Arg72 polymorphism had a stronger ability to bind TAp73 and p53 mutants than the Pro72 form. Intriguingly, the TP53 Arg72 allele was found to be preferentially mutated and retained in squamous tumours of the skin and vulva (Marin et al. 2000). The model that emerges from these studies indicates that the cancer-derived mutant p53 can interfere with the apoptotic function of TAp73, and so make a contribution to tumor development beyond the simple loss of p53 function itself. However, data are not yet available about the physical interactions between these p53 mutants and ΔNp73, Δ2p73 and Δ3p73. Clearly, this information could be extremely important, as mutant p53 might also sequester ΔNp73, and so inhibit its anti-apoptotic function. Again, the cellular context (that is, TA:ΔN ratio of p73) would dictate the outcome.

3. p73 and development.

a) The p73-null mouse.

Unlike those lacking TP63, TP73-/- mice survive post-natally and some live well into adulthood, despite having multiple defects (Yang et al. 2000) (**Fig.27**). Remarkably, given the similarity of the genes, the TP73 knockout phenotype shows no obvious overlap with that of TP63-deficient mice, that we will describe below. TP73-/- mice have malfunctions in fluid dynamics in the central nervous system and respiratory airways, defective neurogenesis, and abnormal reproductive and social behavior.

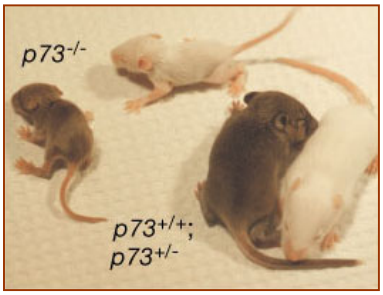


Figure 27. 10 days-old p73-null mice compared to wild-type or heterozygous.

The loss of secretory control in TP73-/- mice is provocative because it suggests a role for p73 in responding to basic physiological stimuli that is arguably more critical to life than tumor suppression.

Specifically, TP73-/- mice develop hydrocephalus, most probably owing to hypersecretion of cerebral spinal fluid by the choroid plexus, and massive inflammation of nasal mucus membranes, primarily owing to hypersecretion by respiratory epithelia. So, two fundamental and highly regulated secretory processes, one controlling intraventricular pressure and the other innate immune responses to microorganisms, seem constitutive in the absence of p73. In addition, the intestinal haemorrhages frequently observed in TP73-/- mice may be the consequence of similar secretory defects in the gastrointestinal tract.

Perhaps one of the more unusual aspects of the TP73-/- phenotype lies in their abnormal reproductive and social behaviour, which, for rodents in general, depends on pheromone signaling (Buck 2000). TP73-/- mice are uncharacteristically uninterested in mating and most other social interactions governed by pheromone signals. The origin of this malaise seems to be a frank absence of pheromone receptor expression on the neurosensory cells of the vomeronasal organ, which express very high levels of TP73 in wild-type mice (Yang et al. 2000). So p73 has a central, albeit poorly defined, function in primitive sensory and signal transduction pathways.

Finally, TP73-/- mice show a severe distortion of the hippocampal formation, a region of the brain considered to be the centre of learning and memory in higher mammals (Yang et al. 2000). The defect here could be attributed to the loss of TP73-expressing Cajal–Retzius cells, which are bipolar neurons found in the marginal zone of the cortex and molecular layers of the hippocampus. Cajal–Retzius neurons have attracted considerable interest because they are one of several populations of pioneer neurons that express reelin, a secreted glycoprotein implicated in development of the cerebral cortex (Frotscher et al. 1998). Although TP73-/- mice lack Cajal–Retzius neurons along the marginal zone of the cortex, they retain other reelin-secreting neurons such as those of the cortical layer, which do not normally express TP73. Interestingly, cortical layering is normal in the TP73-/- mice, indicating that Cajal–Retzius neurons of the marginal zone may function exclusively in regulating hippocampal morphogenesis. Development of the hippocampus, particularly of the dentate gyrus, is especially important, as recent studies have shown that this process

persists throughout adulthood (Eriksson 1998). Stem cells of the granule neurons produce new progeny that require positional cues. So p73 may mediate the survival or proper migration of neurons essential for the constant remodelling of the hippocampus throughout life.

The characterization of the ΔNp73 isoform allowed to understand some discrepancies in the TP73-/- phenotype (Pozniak et al. 2000; Pozniak et al. 2002), as, for example, the developmental death in sympathetic and cortical neurons in the weeks or month after birth, enhanced in the knock-out animals. It has been shown that ΔNp73α and β, expressed in the cortical neurons, are highly potent survival molecules for at least some CNS structures such as the cortex, olfactory bulb and facial motor nucleus. Moreover, it has been proposed that the ventricular enlargement observed in the p73-/- brain was not due to problems with fluid homeostasis, but is a secondary consequence of ongoing cellular loss, a phenomenon also observed in the degenerating human brain.

Hence, the TP73-/- phenotype is complicated by the fact that the two p73 isoforms, TA and ΔN, have been knocked out. In fact, the TA form has been implicated not only in pro-apoptotic pathways, but also in neuronal differentiation signaling : in a neuroblastoma cell line, p73 has been induced by treatment with differencing agents and was then able to induce cell-cycle arrest and neuronal phenotype (De Laurenzi et al. 2000). Moreover, as we've seen, the ΔN isoform is a pro-survival factor, necessary during embryonic stage (facial motor neurons) and for long-term surviving after birth (CNS). An important goal, then, is to generate isotype-specific and conditional knockouts that affect one isotype while preserving the other.

b) Apoptosis in the nervous system.

A general *modus operandi* of metazoan development is the over-production of excess cells followed by an apoptotic culling during later stages of development to match the relative number of cells of different types to achieve proper organ function (Jacobson et al. 1997). Thus, during animal development, numerous structures are formed that are later removed by apoptosis. This enables greater flexibility as primordial structures can be adapted for different functions at various stages of life or in different sexes.

During neuronal development, neurons are in large excess and a great part of them will die by apoptosis, either because they didn't differentiate correctly, or because they compete for the neurotrophin produced by their target cells, a process which is very well known. In fact, in 1949, Viktor Hamburger and Rita Levi-Montalcini described in a seminal paper that the

survival of developing neurons is directly related to the availability of their innervating targets. This laid the foundation for the neurotrophin hypothesis (Purves 1988), which proposed that immature neurons compete for target-derived trophic factors that are in limited supply; only those neurons that are successful in establishing correct synaptic connections would obtain trophic factor support to allow their survival. It was soon discovered that vertebrate neuronal cell death induced by trophic factor deprivation requires the participation of caspases. This was the first functional evidence that trophic factor deprivation activates a cellular suicide programme in vertebrate neurons.

Neurons share the same basic apoptosis programme with all other cell types, they involve the Bcl-2 family proteins and the caspases. However, different types of neurons, and neurons at different developmental stages, express different combinations of Bcl-2 and caspase family members, which is one way of providing the specificity of regulation (Garcia et al.1992). The study of the different knock-out mice allowed to understand their role in neuronal differentiation.

The expression of Bcl-2 is high in the central nervous system during development and is down-regulated after birth, whereas the expression of Bcl-2 in the peripheral nervous system is maintained throughout life (Merry and Korsmeyer, 1997). Although the development of the nervous system in Bcl-2-knockout mice is normal, there is a subsequent loss of motor, sensory and sympathetic neurons after birth (Veis et al. 1993; Michaelidis et al. 1996), suggesting that Bcl-2 is crucial for the maintenance of neuronal survival. Bcl-xL-null mice die around embryonic day 13 with massive cell death in the developing nervous system (Motoyama et al. 1995). Cell death occurs primarily in immature neurons that have not established synaptic connections. Thus, Bcl-xL might be critical for the survival of immature neurons before they establish synaptic connections with their targets. In Bax-deficient mice, superior cervical ganglia and facial nuclei display increased neuron number. Furthermore, neonatal sympathetic neurons and facial motor neurons from Bax-deficient mice are more resistant to cell death induced by NGF deprivation and axotomy, respectively. Thus, the activation of Bax might be a crucial event for neuronal cell death induced by trophic factor withdrawal as well as injury. Apaf-1-null mice die during late embryonic development, exhibiting reduced apoptosis in the brain with a marked enlargement of the periventricular proliferative zone (Cecconi et al. 1998). Thus, Apaf-1 is indispensable in the apoptosis of neuronal progenitor cells.

Neurotrophins generally activate and ligate the Trk receptors (TrkA, TrkB and TrkC), which are cell-surface receptors with intrinsic tyrosine kinase activity. They can autophosphorylate (Barbacid 1995); for instance, after the binding of NGF to TrkA, the receptor phosphorylates several tyrosine residues within its own cytoplasmic tail. These phosphotyrosines in turn serve as docking sites for other molecules such as phospholipase Cγ, phosphoinositide 3-kinase (PI(3)K) (Fruman et al. 1998) and adaptor proteins such as Shc, and these signal transduction molecules coordinate neuronal survival. The removal of NGF results in a decrease in MAP kinase and PI(3)K activities, followed by a series of early metabolic changes including the increased production of reactive oxygen species, decreased glucose uptake and decreased RNA and protein synthesis. In some cells, the removal of NGF results in a slow and sustained increase in c-Jun amino-terminal kinase (JNK) and p38 MAP kinase activities (Xia et al. 1995); in other cells, c-Jun, one of the downstream targets of JNK, is induced and phosphorylated (Estus et al. 1994; Ham et al. 1995). The activation of JNK itself might be necessary, but not sufficient, to induce neuronal apoptosis.

4. p73 stability.

a) Mdm2.

Like p53, p73α seems to be degraded by the proteasome pathway, as proteasome inhibitors, such as LLnL and MG132, increase protein levels. p73α associates with Mdm2 *in vitro* and *in vivo* (Balint et al. 1999; Zeng et al. 1999), and the interaction involves the N-terminal domains of the two proteins, as for p53.

In the case of p73, however, association with Mdm2 does not lead to degradation. The most striking divergence between p53 and p73 resides in the C-terminal, and this part of the protein in p73 is likely to be responsible for the incapacity of Mdm2 to induce degradation.

Nonetheless, p73 binding to Mdm2 still has a functional consequence, as Mdm2 binding inhibits p73α transactivating activity. In fact, Mdm2 is able to bind the cofactor p300/CBP, which is necessary for p73 transactivation activity, and has the same binding site as p73. Mdm2 thus acts as a competitor to p73 for p300/CBP binding (Zeng et al. 1999). The interaction between p53 and p300/CBP is not inhibited by Mdm2, as they use different binding sites in the protein (**Fig.28**). In addition, the competitive binding of p73 to Mdm2 could result in the protection of p53 from Mdm2-induced degradation.

Another difference between p53 and p73 regulation is the cellular localization induced by Mdm2 interaction (Gu et al. 2001). p73 C-terminus is different from the p53 one, and lacks the lysines targeted by the ubiquitination. In fact, Mdm2 expression doesn't induce p73β ubiquitination nor exposition of its NES, but its aggregation and sequestration in the nucleus, in distinct part from the PML dots. Its aggregation is independent of Mdm2 RING-domain, necessary for the E3 ligase activity, and may be also due to the p73 non-functional NES.

More recently, a novel protein, Parc, has been found to regulate the subcellular localization of p53 (Nikolaev et al. 2003). While this protein seems to be responsible for the abnormal ''Parc-king'' of p53 in the cytoplasm in cancers like neuroblastoma, little is known on its possible interactions with p63 and p73, even though the normal nuclear localization of p73 in neuroblastoma seems to exclude this possibility.

As discussed above, p73 binds Mdm2, but it is not degraded in the proteasome, leaving this physical interaction with modest functional effect so far. It is, therefore, tempting to speculate that Mdm2 binding to p73 might produce similar effects to p53: (i) affecting the relative expression of different isoforms, such as ΔNp73, similarly to ΔN-p53, (ii) facilitating the E4 function of p300/CBP as compared to its acetylase activity, indirectly favoring the degradation of p73, possibly during DNA damage or other stresses.

b) SUMO.

p73α, but not β, can be covalently modified by SUMO-1 on the C-terminal Lys627. This modification seems to potentate p73α's proteasomal degradation (Minty et al. 2000), even if Sumoylation is usually associated to protein stabilization. p73β is a much more powerful transactivator than p73α, this could be because the sumoylation of p73α influences interactions with other proteins, such as c-Abl tyrosine kinase. The involvement of SUMO-1 in nuclear bodies has raised the possibility that p73 may interact with PML, thus regulating the localization and the function of the protein.

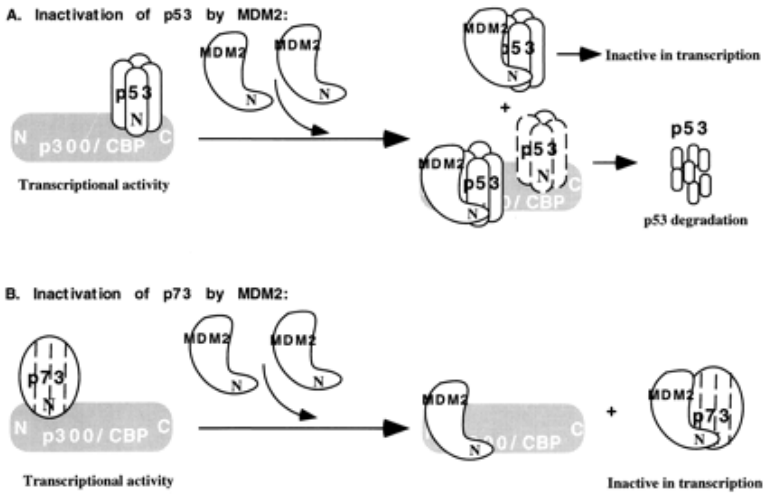


Figure 28. Schematic presentation showing distinct mechanisms by which Mdm2 inactivates p53 (A) and p73 (B). p53 with dashed lines in panel A indicates that this protein may or may not be in the ternary complex. This model does not address the suggested alternative mechanism for p53 inactivation by MDM2, namely, that MDM2 interferes with the binding of other transcription factors, e.g., $hTAF_{II}31$ and $hTAF_{II}70$, to the N-terminal transcription domain of p53. Also, binding of p53 to the N terminus of p300/CBP in the absence of MDM2 is not shown. (Zeng et al. 1999)

5. p73 activation.

a) Phosphorylation.

p73 activity is regulated by several of the same mechanisms as p53, which supports the idea that p73 regulates tumor progression. In addition, novel pathways have been described. p73 is activated by DNA damage, and this is sufficient to trigger cell death, independently of the status of p53. The molecular mechanisms by which p73 is activated involve the presence of the mismatch repair gene MLH1 (Gong et al. 1999; Agami et al. 1999; Yuan et al. 1999; Catani et al. 2002), and a functional and physical interaction between the SH3 domain of c-ABL and the PxxP motif of p73. As c-Abl (Abelson leukaemia) is, itself, phosphorylated and activated by ATM, for example after γ-radiation, ATM might also be included in this pathway. This MLH1–c-Abl–p73 pathway is potentially of great clinical relevance, as it

might explain the response to chemotherapy in the majority (up to two-thirds) of cancers that have mutated or functionally inactivated p53. The activation of p73α or β in response to DNA damage does not seem to require its transcription, instead, it seems to be activated by post-translational modification.

It has been reported that the tyrosine Y99 of p73 is phosphorylated in irradiated cells, but in cisplatin-treated cells, p73 protein stabilization is independent of phosphorylation (Agami et al. 1999; Yuan et al. 1999; Gong et al. 1999).

Moreover, c-Abl activates a group of stress activated protein kinases (SAPK), such as JNK and p38MAP kinase. They are proline-targeted Ser-Thr protein kinases, associated with DNA damage due to UV light or γ-radiation. After cisplatin treatment, p38 alone is also involved and its activation is sufficient to enhance p73 activity. So, p73 can be stabilized through a tyrosine 99 phosphorylation by c-Abl and a Threonine phosphorylation by p38 (Sanchez et al. 2000).

Another kinase, HIPK2 (Homeodomain Interacting Protein Kinase 2), has been found to bind to p73 and to co-localize in the nuclear bodies, which affects its function (Kim et al. 2002).

b) Acetylation.

It has been reported that p73 is acetylated by p300 on carboxy-terminal lysine residues (K321, K327, K331), which determines if p73 will bind on apoptosis or cell cycle promoters, indicating that DNA damage induces several signals to activate p73 (Costanzo et al. 2002). Acetylation results in activation of the p53AIP1 (p53 Apoptosis-Inducing Protein 1) promoter, rather than the CDKN1A promoter, indicating a mechanism that might determine whether cell-cycle arrest or apoptosis is induced.

Whether ΔNp73 is phosphorylated or acetylated is also uncertain, and it remains possible that post-translational modifications enhance the anti-apoptotic function of ΔNp73 to promote survival. Similarly, information is lacking about the interactions of the different splicing isoforms with these kinases and acetylases. It seems likely that the response to DNA damage is highly dependent on the cellular context: determinants might include the relative abundance and modification of each of the p73 isoforms.

c) Oncogenes activation.

Most, if not all, human tumours show deregulation of the E2F family of transcription factors, through loss of the cyclin-dependent kinase inhibitor

$p16^{INK4A}$ (encoded by CDKN2A), overexpression of cyclin D or loss of RB (retinoblastoma). As we have seen before, one of the effects of E2F deregulation is abnormal cell-cycle progression, which contributes to the deregulated growth of tumor cells. E2F1 dependent apoptotic signals are also activated by deregulated E2F, which is thought to be a failsafe mechanism to prevent malignant progression. The apoptotic activity of E2F1 reflects, at least in part, its ability to activate both p53 and p73, even if through quite different mechanisms.

E2F1 regulates p73 levels directly, by binding to E2F-binding sites within the TAp73 promoter (Stiewe et al. 2000; Lissy et al. 2000; Irwin et al. 2000). This transactivation seems to regulate the steady-state protein levels of p73, which fall when cells are induced to exit the cell cycle after serum withdrawal, and re-accumulate after activation of E2F-dependent transcription during S-phase entry. E2F1-mediated apoptosis in mouse embryonic fibroblasts shows a non-additive cooperation between p53 and p73 (Irwin et al. 2000), which is consistent with a synergistic, but independent, contribution of TAp73 and p53 to oncogene-triggered apoptosis in tumor surveillance, and raises crucial questions about the molecular mechanisms of this interaction (**Fig.29**).

The c-Myc oncogene also signals to p73 *in vivo*, and enlists TAp73 to induce apoptosis in tumor cells, possibly by activation of E2F1 (Leone et al. 2001). The interaction between c-Myc and p73 is still controversial. A study reports that c-Myc induces and activates p73 (Zaika et al. 2001), whereas another one shows that c-Myc physically interacts with p73, thereby inhibiting its transcriptional activity (Watanabe et al. 2002). In addition, the c-Myc-binding protein MM1 removes the c-Myc block, thereby potentiating p73 transcription.

As we have seen before for p53, activation of these oncoproteins induces accumulation and stabilization of p53, at least in part through the induction of $p14^{ARF}$, which inhibits Mdm2 to stabilize p53. As Mdm2 doesn't induce p73 degradation but the inhibition of its binding to the transcriptional co-activator p300 and its relocalization, $p14^{ARF}$ doesn't seem to play a role in p73 activation. However, the Mdm2 related protein, MdmX, is able to bind p73, and its over-expression induces p73 re-localization from the nucleus to cytoplasm (Wang X. et al. 2001). As $p14^{ARF}$ is not able to bind MdmX, the different levels of MdmX and Mdm2 may determine the cellular localization of p53 and p73 and their different responses to $p14^{ARF}$.

Finally, the Wilms' tumor-suppressor gene, WT1, is also implicated in the regulation of p53 and p73 (Scharnhorst et al. 2000). We saw first that

p53 represses WT1 transcription. Here, as it happens for p53, the zinc finger of WT1 binds to p73 to inhibit its transcriptional activity.

d) Viral oncoproteins.

The adenovirus E1A protein (Steengenga et al. 1999) and the human T-cell lymphotropic virus 1 (HTLV1) Tax protein (Kaida et al. 2000) bind to and inactivate p53 and p73 (**Fig.29**). By contrast, the adenovirus E1B55, human papillomavirus E6 and simian virus SV40 T-antigen proteins inactivate p53, but not p73 (Marin et al. 1998; Prabhu et al. 1998; Dobbelstein et al. 1998). The adenoviral protein E4orf6 (E4 open reading frame 6) seems to interact with p73 (Roth et al. 1998; Higashino et al. 1998), although this result is not unanimously accepted (Roth et al. 1998). The differential viral recognition of p53 and p73 indicates that p53 and p73 might have different effects on viral replication and transformation.

6. p73 transcriptional activity after stress.

p73 can elicit cell-cycle arrest (Kaghad et al. 1997; De Laurenzi et al. 1998), apoptosis (Jost et al. 1997), neuronal differentiation (De Laurenzi et al. 2000) and epidermal differentiation (De laurenzi et al. 2000). Although the molecular mechanisms by which p73 triggers cell death have not been described in great detail, there is an overall assumption that p73 uses pathways that are almost identical to those described for p53. In keeping with this idea, p73 is able to drive the transcription of several apoptotic effectors that are also induced by p53, including Bax, p53AIP1 (Steengenga et al. 1999; Zhu et al. 1998; Prives et al. 2001), PERP, NOXA (Flores et al. 2001), PUMA and CD95 (**Fig.30**). Whether a simple increase in the expression of these proteins would be sufficient to trigger apoptosis, or whether other events are required, has yet to be shown.

Despite the similarities between p73 and p53, it is also becoming evident that p73 has activities that are not shared by p53. For example, p73β can enhance WNT/ß-catenin signaling through mechanisms that are not shared by p53 (Ueda et al. 2001), and p73α has been shown to activate promoters that do not contain consensus p53-binding sites (Takagi et al. 2001). Indeed, p73β was shown to induce expression from the CDKN1B promoter, which encodes the cyclin-dependent kinase inhibitor KIP2 (also known as p57; encoded by CDKN2C), through a mechanism that allows co-regulation of several genes within the Beckwith-Weidemann Syndrome imprinted cluster (Balint et al 2002). p53 does not share this activity,

indicating that p73 has a specific role in regulating the expression of genes that are important for normal development.

p73 might also contribute indirectly to the activation of apoptosis by modulating p53 function. The physical binding of p73 to Mdm2 might sequester Mdm2 and lead to an increase in the steady-state level of p53 and, therefore, of its apoptotic function. There is also strong evidence that the ΔNp73 isoforms can protect cells from apoptosis (Pozniak et al. 2000; Grob et al. 2001). This inhibition seems to be elicited by directly inhibiting the pro-apoptotic activity of TAp73 and p53 by means of the two distinct mechanisms (sequestration and competition).

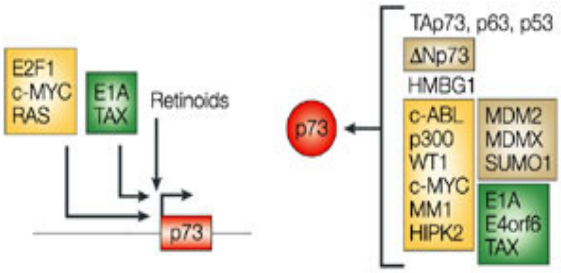


Figure 29. p73 activation.
p73 may be transcriptionaly activated by oncogene (E2F, c-MYC) or by viral oncoproteins. Moreover, p73 can be activated by post-translational modifications such as phosphorylation (c-ABL), acetylation (p300), sequestration (ΔNp73, WT1 and maybe SUMO). (Melino et al. 2002)

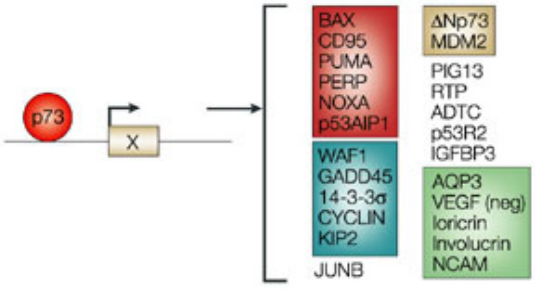


Figure 30. p73 transcriptional activity.
As p53, p73 target genes are involved in apoptosis (in red), cell-cycle arrest (in blu) or in its own regulation (in brown). Moreover, p73 is also involved in neuronal and epithelial differentiation (in green). (Melino et al. 2002)

C. p63 : the last characterized, but the oldest member of the family.

1. Characterization.

The story was made more complex with the discovery of yet another p53 family member, p63 (also called KET, TP51, p40, p73L, or AIS), which, despite its sequence homology, has its own peculiarities that placed it apart from p53 and p73 (Yang et al. 1998; Schmale et al. 1997; Osada et al. 1998; Senoo et al. 1998; Trink et al.1998). For instance, its chromosomal location at 3q27 is frequently amplified in cancers rather than deleted, suggesting the possibility that p63 might be acting as a proto-oncogene. In addition, the expression of p63 in the basal cells of many epithelial tissues, including skin, breast, prostate and urothelia, suggested functions other than the contingency actions of p53 (Yang et al. 1998). Although p63 is able to promote the expression of several of the same genes as p53, very few p63 mutations have been seen in tumors, and whether the dominant-negative isoforms of p63 act to promote cancers by competing with p53 or p73 is not established.

p63 presents the same structure as p53 and p73, as we have seen before (**Fig. 21, 22 and 31**) : the two transcription initiation sites and alternative splicing routes, yielding at least six different p63 isoforms, the transactivation domain (TA), the DNA-binding domain and tetramerization domain (Iso). The α isoform contains in addition the sterile α motif (SAM) domain and a transactivation inhibitory domain (TID) at the C-terminus whom role will be detailed below.

a) The ΔN isoform.

As ΔNp73, ΔNp63 is able to block the p73, p63 and p53 transcriptional activity. But the most interesting information about these two isoform may come from their expression pattern : in fact, as we've seen first, in both the developing and adult mouse brain, evidence is strong that ΔNp73 isotypes are the main expression products, yielding protein levels 20-fold greater that those of the TA-p73 isotypes (Yang et al. 2000; Pozniak et al. 2000). Likewise, evidence that ΔNp63 is key player *in vivo* came from the initial characterization of TP63 expression (Yang et al. 1998). Antibodies to p63 showed intense staining of the basal, or progenitor, cell layers of a wide range of epithelial tissues, including the epidermis, cervix, urogenital tract, prostate, myoepithelium of the breast and other glandular tissues (Yang et al. 1998) (**Fig.32**).

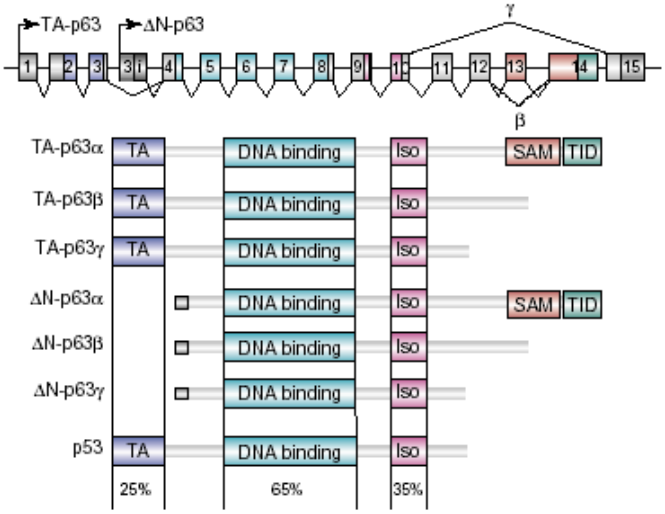


Figure 31. p63 gene structure and transcripts.

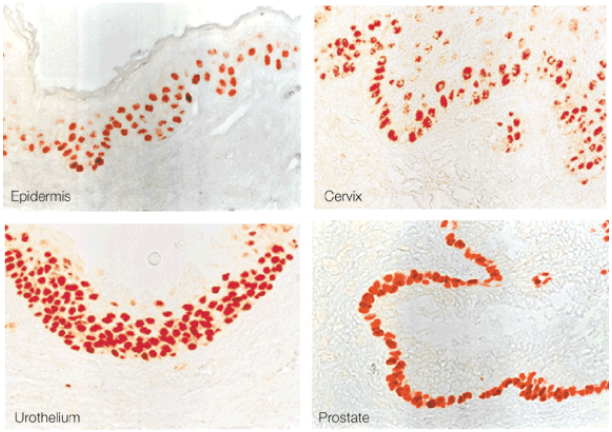


Figure 32. ΔN isotypes are predominantly expressed *in vivo.*
Immuno-histochemical staining with a monoclonal antibody against p63 reveals high levels of p63 protein in human epithelial tissues, as indicated. Protein-size and RNA analysis further indicate that ΔN-p63 isotypes are the predominant proteins in these cells (Yang et al. 1998). (Yang and McKeon 2000)

Together with analyses of protein size and RNA expression, these findings showed that ΔN products account for virtually all p63 protein expression in the epithelial tissues examined and in keratinocytes, for instance, ΔNp63 is present at five times the concentration of p53, suggesting the possibility that these cells are functionally p53-deficient under basal conditions.

Moreover, the data from TP73 knockout mice indicates that ΔNp73 may suppress p53 activity in certain cell populations such as sympathetic neurons (Pozniak et al. 2000), increasing their survival. In another study, immature keratinocytes exposed to ultraviolet radiation showed a rapid loss of ΔNp63 coincident with the stabilization of p53 (Liefer et al. 2000). Whether this loss of p63 is provoked by stabilized p53 or a separate but coordinate response to DNA damage remains an unanswered but intriguing question.

The N-truncated isoforms play an important role not only in development, but also in tumor formation, if the different isotypes are deregulated as it happens in neuroblastoma for ΔNp73 and as it may explains the 3q27-ter locus amplification in some carcinomas. The regulation of all these isoforms levels is therefore of very important interest, as it may depend on differentiation signals, DNA damage or stress and involve differential transcriptional regulation or protein stability.

b) <u>The C-Terminal domain.</u>

A variety of signals regulate the C-terminus of p53 which negatively regulates the protein's function. In the same way, the p73 and p63 responses seem to be regulated by their respective C-termini, though these responses are predictably different in the various splice variants presents in these proteins. Serber et al. (2002) identified a new 71 amino acid domain in the C-terminus of the p63α protein, the TID (Transactivation Inhibitory Domain), which is able to inhibit the transactivation domain. In his model, a molecule of TAp63α is folded and inactivated by the interaction transactivation domain /TID. This could explain why TAp63α is a less efficient activator than TA-p63β. In the same way, the ΔN-isoforms, which lack the TA domain, are not able to equally inhibit the TAp63 proteins. In fact, ΔN-p63α has a stronger dominant negative effect than the β and γ forms.

Moreover, the SAM-containing proteins are more stable, as the TID domain, bound to the TA, hides the sites used by the degradation pathway. Therefore, the p63 proteins are not only regulated in their transcriptional

activity but also in the stability levels of the various isoforms. The complexes between TAp63 isoforms and ΔN-p63α or the TA/TID intramolecular folding increase the half-life of the proteins and keep them in an inactive form. This pool of protein is ready to be used in case of a stimulus such as a developmental signal. The SAM domain may play a role in the activation of the protein, binding other activating partners, such as kinases which could phosphorylate the TA or TID domain and thus open the protein in an active form.

The high sequence homology between p63 and p73 predicts that these results could possibly be relevant also for p73 regulation, as it has been first evoked by the enhancement of TAp73α transactivity after deletion of its C-terminal domain (Ozaki et al. 1999). However, according to Lee and La Thangue (1999), p73β is much more active because more stable. p73β, but not α, increases after LLnL treatment, suggesting that the SAM domain of the α form negatively regulates this degradation pathway. This paradox could be explained by some small differences in the two protein sequences, or by different systems used to explore the protein's stability.

2. p63 and development.

a) The p63-null mouse.

Mice deficient in p63 show striking epithelial defects, including a complete absence of skin, hair, mammary tissue, prostate, lachrymal glands and salivary glands, as well as pronounced alterations in epithelia of the urogenital tract, tongue and stomach (Mills et al. 1999; Yang et al. 1999). Severe limb truncations and craniofacial malformations are also seen (**Fig.33**).

Many of the affected sites are tissues that, in normal animals, show epithelial stratification, with immature, progenitor cells along the basal layer and more differentiated cells above. For example, even developing limb buds in the embryo are covered by an epithelium that later stratifies to form the apical ectodermal ridge, amulti-layered structure essential for growth and patterning of the underlying mesenchyme (Johnson and Tabin, 1997).

The affected tissues in the TP63 knockout mouse are generally characterized by absence of cells (**Fig.34**). These phenomena has been first interpreted as aborted epithelial differentiation (Mills et al. 1999), but it seems that the p63-/- epithelial cells are able to differentiate and that the p63 function would be in maintaining the basal, progenitor cell population that

underlie the capacity of the tissue to develop and regenerate (Yang et al. 1999).

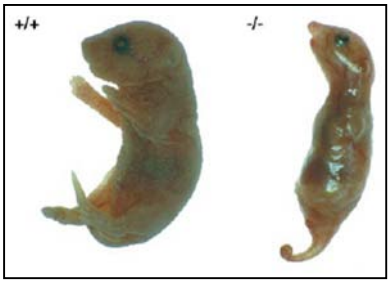


Figure 33. p63-deficient newborn mouse phenotype. (Yang et al. 2000)

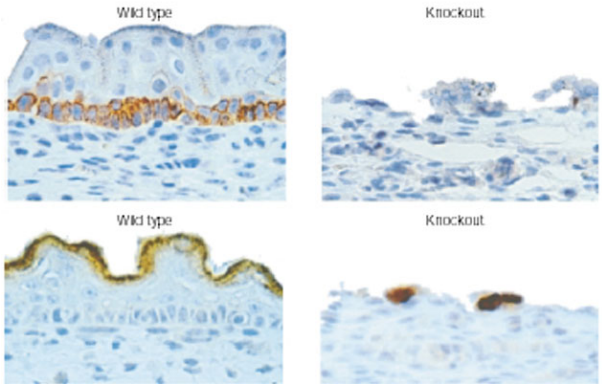


Figure 34. Epithelial defects in p63-deficient mice: hints for a role in stem-cell regeneration?
a) Skin sections of late-stage *TP63* knockout and wild-type embryos were stained with antibodies to keratin 5, a marker of basal, proliferative cells of the epidermis. Wild-type sections show strong staining, whereas the tattered epidermis lacking *TP63* shows no keratin 5 expression, indicating a lack of basal cells. **b**) Residual epithelial cells along the denuded surface of *TP63* knockout embryos show positive staining for loricrin, a marker of differentiated, mature cells in cornified layers of a stratified epithelium. Both wild-type and knockout cells stain for loricrin, indicating that *TP63* knockout epidermis undergoes differentiation processes.

b) p63-linked human syndromes.

Simultaneous with the analysis of the p63 gene in mice, investigations were underway to discover genes responsible for the ectodermal dysplasia and limb abnormalities of a large Dutch family; the genes mapped very close to the p63 gene on chromosome 3q27–q28 (van Bokhoven et al. 1999). The constellation of phenotypic features in this family was recognized as a separate clinical entity, denoted LMS (Limb-Mammary Syndrome), related to the more common EEC (Ectrodactyly-Ectodermal dysplasia-Clefting) syndrome. This latter syndrome, described as early as 1804 by Eckhold and Martens had three cardinal features reflected in the name: ectrodactyly, also known as split hand/foot or lobster claw deformity; ectodermal dysplasia affecting hair, skin, nails, teeth and sweat glands; and cleft lip with or without cleft palate (CL/P). Other symptoms are variable and include lacrimal duct abnormalities, urogenital problems, conductive hearing loss, facial dysmorphism, chronic respiratory infections and mental retardation.

Taken as a whole, these syndromes showed many of the features of the defects seen in the p63-null mice, especially with the limbs and epithelial tissues, although the human syndromes appeared, in general, less severe in all aspects. A limited linkage analysis with markers from 3q27–q28 in several EEC syndrome families indicated that LMS and EEC syndrome were indeed allelic. In the EEC cases, p63 is mutated in “hot-spot” sites in the DNA Binding Domain, and in the LMS, a frame shift mutation affects the C-terminal domain and encodes a p63 lacking the SAM and TID. In rapid succession, nine unrelated patients with EEC syndrome were shown to have heterozygous mutations in the p63 gene (Celli et al. 1999). The excitement generated by this discovery was increased by the fact that a large number of human syndromes exist involving limb development and/or ectodermal dysplasia whose analysis could shed light on the p63 gene or regulators of p63. They include: LMS; the Hay–Wells syndrome, or AEC (Ankyloblepharon-Ectodermal dysplasia-Clefting) syndrome, which is due to point mutations in the SAM domain; the ADULT (Acro-Dermato-Ungual-Lacrimal-Tooth) syndrome, due to a particular mutation in the DNA Binding Domain or in the ΔNp63 exon3’; and the isolated SHFM (Split Hand/Foot Malformation) which is genetically more heterogeneous. Hence, all of these disorders are the consequence of a mutation at a single TP63 allele and, if one can extrapolate from the mouse knockout models, are not the result of haplo-insufficiency. Indeed, rare patients with a heterozygous deletion at 3q27–q29, encompassing the TP63 gene, display no signs of EEC syndrome

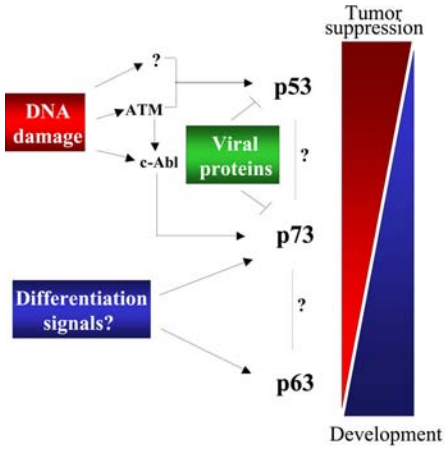
(van Bokhoven et al. 2001). Therefore, the possibility must be considered that these mutations have dominant-negative or gain-of-function effects.

In contrast to the missense mutations of DNA binding hotspot residues, frameshift mutations found in LMS and EEC syndrome result in premature truncations of the p63 alpha isoforms. Again, the consequences of such mutations will vary depending on the relative contributions of TAp63α or ΔN-p63α to the disrupted developmental program. In the case of TA-p63α, which appears to lack transcriptional activity owing to the C-terminal TID, these frameshifts remove the TID and therefore restore transactivation potential to these molecules. By contrast, the loss of the TID domain from the ΔNp63α isoform might prevent these molecules from acting as *trans*-dominant inhibitors of associated TA-p63 isoforms. However, the loss of the TID domain from the ΔN-p63α isoform should not prevent its binding to DNA and therefore should spare this competitive aspect of its repressor function. In this regard, the biological consequences of the SAM domain mutations found in the AEC syndrome are uncertain. The SAM domain itself might be a means of relieving the actions of the adjacent TID domain, presumably as a result of recruiting an as-yet-unknown docking protein. The loss of SAM domain function due to the AEC mutations could result in a TA-p63α isoform that is never able to be activated, and therefore assumes permanent repressor activity. For ΔNp63α, the TID-mediated, *trans*-repressor activity would also be constitutive and therefore result in an overall super repressor and an overall decrease in p63 transcriptional activity.

RESULTS

PRESENTATION OF THE RESEARCH

Hence, the two recently discovered p73 and p63 appear more and more different from their well-known relative, p53. Even if p73 and p63 present a high sequence homology with p53, and therefore are potentially able to act as pro-apoptotic and tumor-suppressor proteins, they present a considerable complexity of intra-molecular regulation. In fact, they both give rise to several C-terminal and N-terminal splicing variants and each of these isoforms may play a different role and/or regulate the others. Moreover, in the light of 6 year-study, p63 and p73 appear to be phylogenetically much older than p53 and - this is particularly true for p63 - are also involved in development and differentiation (See model below).



The ΔN p73 and p63 isoforms seem to be involved in differentiating processes as they are, at least in the mouse, the major isoforms expressed in brain and epithelium, respectively. Their absence in likely to be responsible for the developmental defects observed in the TP73 and TP63 knock-out mice. Moreover, as they act as dominant negatives, inhibiting p53 and TA isoforms activities, they are likely to be implicated in tumor development, for example by deregulation of the ratio TA/ΔN.

When we started this work, the murine ΔNp73 isoforms were recently characterized, and we decided to investigate on a possible human homolog. The aims of the work were (i) the characterization of the human ΔNp73 isoforms, the study of their expression and activity; (ii) the ΔNp73 post-translational modifications, especially after DNA damage.

Cloning of human ΔNp73 isoforms

As we have seen before, the p73 gene shows a number of different splice variants clustered at the 3' end of the gene that result in a number of different C-termini. At least in mice, p73 also shows two different N-termini, one containing (TA forms) and one lacking (ΔN forms) the transactivation domain. The latter derives from the usage of a different ATG, located in an additional exon (exon 3'), driven by a second promoter (**Fig.1A**).

In order to clone the human homologues of the mice ΔNp73 isoforms we performed a BLAST search in the genebank database using a sequence from mice exon 3' (y19235). This search allowed us to identify a genomic clone (AL136528) containing the entire human p73 gene. We were therefore able to design forward primers within human exon 3' and amplify the entire coding sequence of ΔNp73α, β and γ [ΔNp73α (AY040827); ΔNp73β (AY040828) ΔNp73γ (AY040829)]. The human ΔNp73 isoforms are highly homologous to the mouse ΔNp73 isoforms (**Fig.1B**). Interestingly, the sequence of exon 3' contains two different in frame ATGs and translation can start with either one (**Fig.1B**). The existence of two different translation start sites is confirmed by in vitro translation of a ΔNp73 construct (**Fig.1C**) and by western blot analysis of over-expressed (**Fig.1C**) and endogenous p73 (**Fig.3B**). In addition we confirmed the use of both ATGs by in vitro translating ΔNp73 cDNAs in which either one of the two ATGs was mutated, showing that only one protein band was present (**Fig.1D**).

Moreover, fluorescence microscopy of cells transfected with TAp73α and ΔNp73α shows that both forms are localized in the nucleus (**Fig.2**).

Expression pattern of human ΔNp73 isoforms

RT-PCR of the entire open reading frame of p73 using forward primers specific for either TA or ΔN, followed by a nested PCR spanning exons from 8 to 14 (see Introduction Fig.21), shows that mRNA for nearly all C-terminous variants exists as both TA and ΔN variants (**Fig.3A**). Western blot analysis with an antibody directed against the C-terminus of p73α, that recognises only the α isoforms, shows that ΔN and TAp73 proteins can be detected only in a small subset of different cell lines tested and that with the exception of HaCaT cells, the TA isoforms were the most represented (**Fig.3B**).

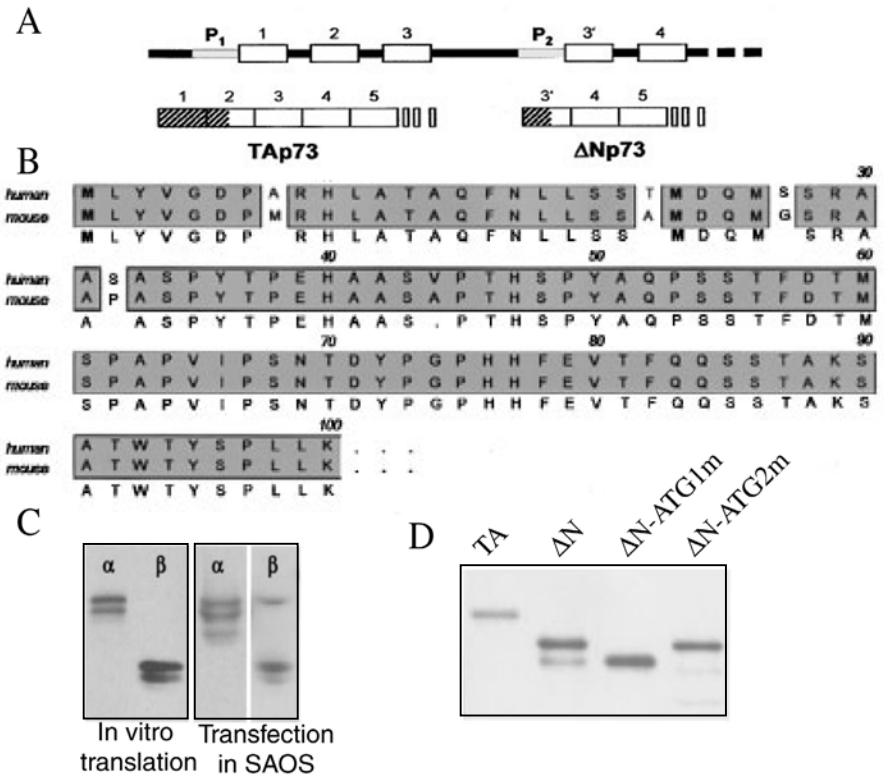


Figure 1. ΔNp73 gene structure and aminoacid sequence. (**A**) Schematic representation of the 5' end of the p73 gene giving rise to TA and ΔN variants. Distances are not proportional to genomic distances. White boxes represent exons (numbers are indicated above in arabic), dark grey shading represents 5' untranslated regions. The light grey shading indicates the two promoter regions: P_1 (coding for TAp73) and P_2 (coding for ΔNp73). (**B**) Alignment of mouse and human ΔN amino-termini, only four aminoacids differ in this region between the two sequences, one of which is encoded by exon 3'. Initial methionines are in bold. (**C**) Western blot of in vitro translated (left) and over-expressed (right) ΔNp73α (α) and ΔNp73β (β) proteins, showing that two different forms derived from two different ATGs exist. 30 μg of protein extract were separated and blotted and revealed with a anti p73 antibody, as indicated in the experimental section. (**D**) Western blot analysis of in vitro translated proteins showing that the double band observed in ΔN expressing cells is due to the use of different ATGs. (TA) TAp73α; (ΔN)ΔNp73α; (ΔN-ATG1m) ΔNp73α carrying an a235c mutation removing the first ATG; ; (ΔN-ATG2m) ΔNp73α carrying an a301c mutation removing the second ATG.

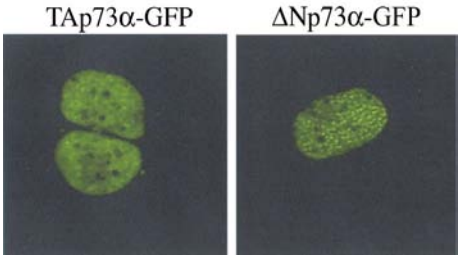


Figure 2
Fluorescence microscopy examination of Saos-2 cells transfected with TAp73α-GFP and ΔNp73α-GFP plasmids showing that both forms are expressed in the nucleus. Cells were analysed at 490 nm with a CDD camera.

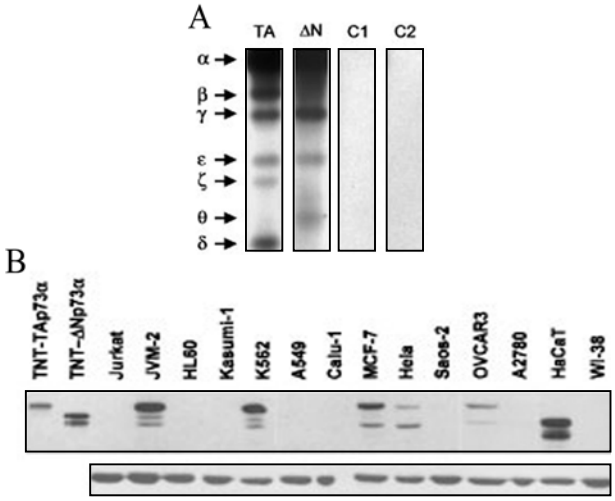


Figure 3. Expression of the different p73 isoforms.
(**A**) RT PCR demonstrating the existence of all different C-termini isoforms with both TA and ΔN aminotermini. RT-PCR products of the open reading frames of both TA and ΔN forms, obtained using specific forward primers, were re-amplified using nested primers spanning from exon 8 to exon 14, common to both p73 variants, then blotted and detected with a probe spanning from exon 8 to 10. Controls omitting the first amplification (C1) or omitting RNA in the amplification mix (C2) are shown. A representative experiment of three performed is shown. (**B**) Western blot of protein extracts from different cell lines, TNT-TAp73α and TNTΔNp73α indicate in vitro translated TA and ΔNp73α respectively. Bottom lane represents actin control. 30μg of proteins extract from each indicated cell line was separated electophoretically, blotted and revealed with an anti-p73 antibody as described in material and methods. A representative experiment of three performed is shown.

Since it has been previously reported by us and others (Kaghad et al. 1997, De Laurenzi et al. 1998) that in most tissues and cell lines p73 is expressed at very low levels, we developed a very sensitive quantitative real time RT-PCR to evaluate the expression levels of TA and ΔN isoforms in different tissues and cell lines. Our results summarised in **table 1** show that differently from what was previously reported for mouse p73, in human tissues and cell lines TA isoforms are the most represented. Our results also suggest that foetal tissues express 10 fold more p73 (both TA and ΔN) than the corresponding adult tissues, underlining its important role in development. Interestingly, breast and ovary show the highest expression levels in adult tissues. In all cases TA isoforms are more expressed than ΔN in the same sample.

ΔNp73 isoforms function as dominant negatives

Mouse p73 and p63 ΔN isoforms have been shown to act as dominant negatives (Yang & McKeon 2000; Yang et al. 2000; Pozniak et al. 2000), thus regulating the activity of the full length family members. In order to show that the human ΔN isoforms we have cloned are also functioning as dominant negatives on p73 and on its homologue p53, we cloned the different ΔN isoforms into a mammalian expression vector (pCDNA-3) under the control of the CMV promoter and performed co-transfection experiments.

As shown in **figure 4**, ΔNp73 is capable of blocking the ability of either p73 or p53 to transactivate the p21 promoter in a dose dependent manner (**Fig.4A and 4B**). Experiments were also performed with plasmids expressing ΔN isoforms starting with either of the two ATGs, and similar results were obtained, showing that both forms were able to block p53 and TAp73 induced transcription at comparable levels. All experiments were performed in SAOS-2 cells, which are p53-null, osteosarcoma cells,.

We also investigated the ability of ΔNp73 to interfere with a highly important cellular function of p53, namely the ability to induce apoptosis. In fact, ΔNp73 is able to significantly reduce the apoptosis induced by TAp73 or p53 over-expression when co-transfected into SAOS-2 cells (**Fig.5**).

From these data, it appears clearly that ΔNp73 is able to block both p73 and p53 activity. In order to rule out whether ΔN isoforms block p53 and TAp73 by competing with the binding to DNA or by forming inactive complexes with p53 and TAp73 we produced mutants of the DNA binding

Normal tissues	TA/7s	ΔN/7s	TA/ΔN
Adult skeletal muscle	9.48×10^{-7}	8.67×10^{-8}	10.9
Adult breast	1.06×10^{-5}	4.25×10^{-6}	2.5
Adult ovary	8.18×10^{-6}	6.29×10^{-7}	13.0
Adult kidney	1.22×10^{-6}	1.05×10^{-7}	11.6
Adult colon	2.21×10^{-6}	1.39×10^{-7}	15.9
Adult stomach	1.77×10^{-6}	4.13×10^{-8}	42.9
Adult liver	4.35×10^{-6}	2.61×10^{-7}	16.6
Adult lung	8.76×10^{-6}	6.01×10^{-7}	14.6
Fetal liver	2.28×10^{-6}	1.71×10^{-7}	13.3
Fetal lung	5.00×10^{-5}	4.42×10^{-6}	11.3
Fetal brain	2.70×10^{-6}	9.15×10^{-7}	2.9
Cell lines			
EPI	1.63×10^{-6}	3.47×10^{-8}	47.1
HepG2	5.65×10^{-5}	6.10×10^{-7}	92.5
LAN5	5.93×10^{-5}	2.26×10^{-6}	26.3
SK-N-BE	1.44×10^{-7}	1.68×10^{-8}	8.6
SH-Sy5y	1.47×10^{-5}	3.31×10^{-7}	44.5
SK-N-SH	4.09×10^{-5}	1.37×10^{-6}	30.0
SK-N-AS	2.04×10^{-8}	ND	–
WI-38	1.93×10^{-7}	2.03×10^{-8}	9.5
HaCat	2.49×10^{-6}	1.05×10^{-6}	2.4
A2780	3.35×10^{-5}	8.10×10^{-7}	41.3
OVCAR3	3.78×10^{-5}	2.15×10^{-6}	17.6
Saos-2	9.03×10^{-7}	1.72×10^{-8}	52.4
Hela	3.47×10^{-7}	6.06×10^{-8}	5.7
MCF-7	1.77×10^{-5}	1.43×10^{-7}	123.8
Calu-1	5.02×10^{-6}	5.24×10^{-8}	95.8
A549	6.77×10^{-7}	2.87×10^{-8}	23.6
K562	2.94×10^{-5}	6.33×10^{-7}	46.4
Hl60	4.84×10^{-7}	4.34×10^{-8}	11.2
JVM-2	3.37×10^{-4}	4.07×10^{-6}	82.8
Jurkat	1.33×10^{-9}	6.30×10^{-9}	0.2
Kasumi-1	ND	ND	–

Table 1. mRNA expression of TAp73 and ΔNp73 determined by real-time quantitative RT-PCR.

Values are expressed as a ratio between the number of transcripts measured for TAp73 or ΔNp73 and 7s ribosomal RNA in 25ng of total RNA. The same RNA sample is used for all three amplification reactions (TA, ΔN and 7s) by real-time RT-PCR. The table reports the mean of the results obtained over three distinct measurements. The standard mean error was always within 10% of the measured value. ND indicates cases where no amplification of the gene was obtained.

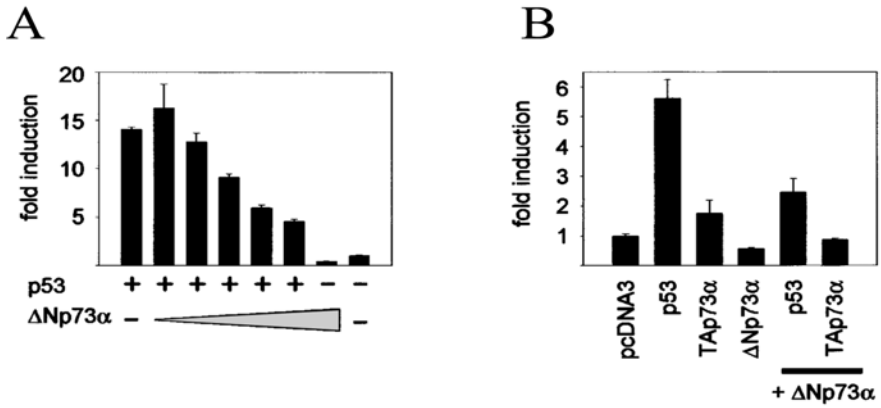


Figure 4. ΔNp73α blocks p53 and p73 transcription activity on p21 promoter in SAOS-2 cells.
(**A**) Luciferase assay with Saos-2 cells transfected with 900 ng of p21-luc, 20 ng of p53 and increasing concentrations (from 30 to 180 ng) of ΔNp73α. The histogram reports the results of three experiments performed. Results are expressed as fold induction over the reporter alone (**B**) Luciferase assay with Saos-2 cells transfected with 1 μg of p21-luc, and 40 ng of p53, or TAp73α alone or in combination with 360 ng of ΔNp73α. The histogram reports the results of five experiments performed. Results are expressed as fold induction over the reporter alone

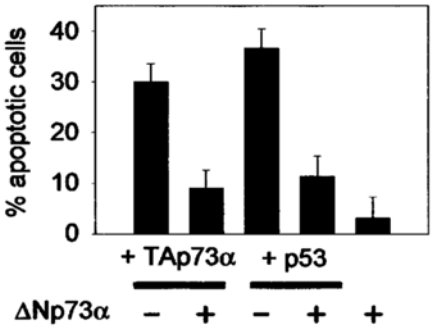


Figure 5. ΔNp73α blocks p53 and p73-induced apoptosis in SAOS-2 cells.
Evaluation of hypodiploid apoptotic events after PI staining of Saos-2 cells transfected with 200ng of p53, or TAp73α alone or in combination with 200ng of ΔNp73α. The histogram reports the results of five distinct experiments performed.

domain of both TA and ΔNp73 isoforms. We mutated the same arginine residue in the DNA binding domain into a histidine (R292H for TA; R244H for ΔN). This mutation is known to determine loss of DNA binding activity of TAp73 and p53, transforming them into dominant negatives that inhibit the corresponding wild-type forms (Jost et al. 1997). Our results show that while both R292H-TAp73 and R244H- ΔNp73 are still capable of inhibiting TAp73 transcription they both loose the ability to interfere with p53 activity (**Fig.6A**). This result strongly suggest that p53 inhibition is achieved through the competition for the binding to specific responsive elements while p73 is blocked also through a hetero-oligomerization between the TA and the ΔN forms (**Fig.6B**).

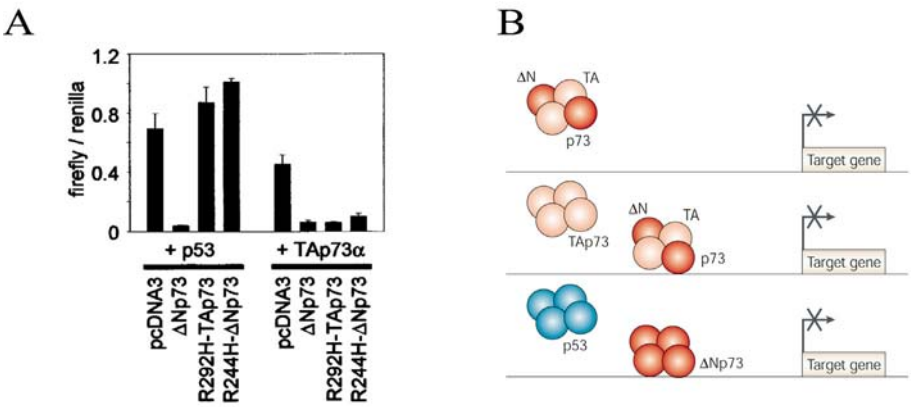


Figure 6. ΔNp73α blocks p53 activity by competition and TAp73 by sequestering it. (**A**) Luciferase assay with Saos-2 cells co-transfected with 800ng of a p53 responsive promoter with 20ng of either p53 or TAp73α expressing plasmids together with 180ng of either wild type ΔNp73α (ΔN) or DNA binding domain mutants of ΔNp73α (R244H-ΔNp73) or of TAp73α (R292H-TAp73) or the empty vector (pCDNA3). The histograms reports the results of three experiments, expressed as fold induction over the reporter co-transfected with p53 or p73. (**B**) Schematic model of ΔNp73α inhibition on TAp73 and p53. ΔNp73 may inhibit TA by sequestering it or forming a less active heterodimer. ΔNp73 may compete with p53 for the same promoter responsive element.

Cloning and characterisation of ΔNp73 promoter

As shown by western blot analysis in Figure 3B, endogenous ΔNp73 is not detectable in numerous cell lines, such as SAOS-2 cells. Nevertheless, while controlling by Western blot the p73 expression in transfected SAOS-2 for Luciferase Assays and measurement of apoptosis (**Figures 4, 5 and 6**), surprisingly we saw an endogenous induction of ΔNp73α (**Fig.7**).

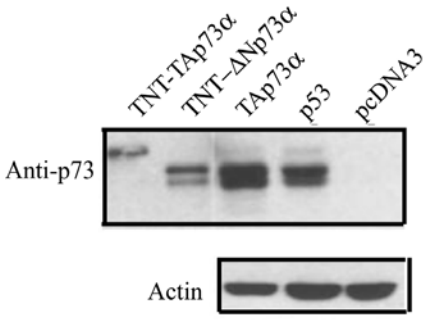


Figure 7. Induction of ΔNp73 protein by p53 and TAp73.
Western blot analysis of SAOS-2 cells after transfection of p53 family members. An antibody specific for p73α (detecting TA and ΔN forms) was used. Specific induction of ΔNp73 is shown by transfection of p53 and TAp73α. TAp73α transfected cells are not shown because the over-expressed protein migrates very close to the ΔNp73 rendering detection impossible. In vitro transcribed and translated TAp73α (TNT-TAp73α) and ΔNp73α (TNT-ΔNp73α) proteins are shown. The two ΔNp73 bands result from the use of the two different start codons (Fig. 1C). Western blot using an antibody detecting the HA-tag fused to the proteins was used as a transfection control. Equal loading is shown with an anti-actin antibody in the lower lane.

In order to determine if this induction was due to ΔNp73 protein stabilization or to transcriptional activation, we first decided to clone and characterize ΔNp73 promoter.

To confirm that the transcription of ΔNp73 isoforms is driven by a different promoter located upstream of exon 3', we first determined the beginning of the messenger RNA by 5' race and RNA protection assay and then cloned a genomic fragment upstream of the transcription start site by

PCR using primers designed on the genomic sequence previously described (AL136528).

As shown in **figure 8A** the messenger RNA contains 235 nucleotides of 5' untranslated RNA which is not interrupted by introns and a TATA box is in position -25. We cloned an approximately 2 kb fragment (from nucleotide 43580 to nucleotide 45728 of sequence, AL136528) of the 5' flanking sequence into the pGL3-basic vector upstream of the luciferase gene (construct A), and tested its ability to drive transcription of the luciferase gene. We performed Luciferase assays in 5 different cell lines (**Fig.8B**) and obtained results correlated to the real time PCR data (**Table1**), indicating that this fragment drives the expression of the luciferase gene when transfected into cell lines expressing high levels of ΔN but not in those that are negative or low expressing. Hence, the 2kb fragment probably contains all the responsive elements necessary to ΔNp73 expression regulation

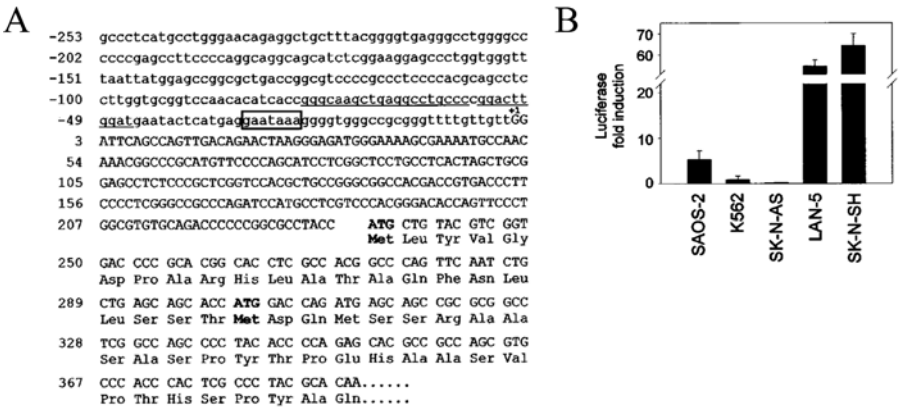


Figure 8. ΔN promoter structure and characterisation.

(**A**) Partial sequence of the ΔN promoter region, transcribed sequence is capitalised, the first transcribed nucleotide is numbered as +1. The box indicates the putative TATA-box. The two initiation codons are in bold. p53 consensus sequence is underlined. (**B**) Luciferase assay of cell lines expressing different amounts of ΔNp73 mRNA show comparable induction of the ΔN promoter: low expressing Saos-2, K562 (left) and high expressing LAN-5 and SK-N-SH (right) cells transfected with a vector containing a luciferase reporter gene (pGL3basic) under the control of a 2 kb fragment of the ΔN promoter (construct A) are shown. The histogram reports the results of five experiments performed expressed as fold induction over cells transfected with the pGL3basic vector alone. Transfection of SK-N-AS cells that have undetectable levels of ΔNp73 are also shown and no induction of the promoter is observed.

p53 and TAp73 regulate ΔNp73 expression.

In order to study whether p53 and TAp73s act on ΔNp73 promoter we performed luciferase assays co-transfecting the ΔNp73 promoter together with p53 and TAp73 in SAOS-2 cells. **Figure 9** shows that ΔNp73 promoter is strongly induced by both p53 and TAp73 α, β and γ.

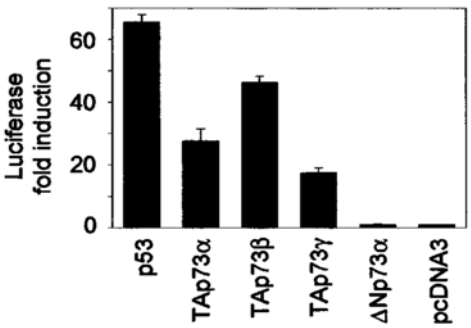


Figure 9. Over-expressed p53 and TAp73 are able to activated ΔNp73 in SAOS-2 cells
Luciferase assay of SAOS-2 cells co-transfected with ΔNp73 promoter construct and p53 or TAp73 α,β, and γ or the empty vector (pcDNA3). Values are expressed as fold induction over control transfected cells. Values are a mean of three experiments.

Induction of ΔNp73 transcripts				
	Transcripts		Fold induction	
Transfected with:	TAp73/7s	ΔNp73/7s	TAp73	ΔNp73
pcDNA3	3.45×10^{-5}	6.19×10^{-7}	1.0	1.0
p53	8.82×10^{-4}	2.24×10^{-3}	25.6	3620.0
TAp73α		1.35×10^{-3}		2177.8
TAp73β		3.57×10^{-3}		5763.5

Table 2. Real-time RT-PCR experiments specific for TAp73 and ΔNp73.
Transient transfection of p53, TAp73a or TAp73b in Saos-2 cells were performed by electroporation. p73 transcript numbers were normalized using 7s ribosomal RNA as a house keeping gene. Fold induction was calculated using the normalized transcript numbers.

Sequence analysis of ΔNp73 promoter region revealed the presence of a single p53 responsive element, which consists of 3 half-binding sites, located at position -47/-78 from the cap-site (**Fig.10A**). This suggested that expression of the ΔNp73 isoforms could be regulated by p53 and by TAp73. To confirm that p53 and TAp73 directly induce ΔNp73 acting on the p53 responsive element identified at position -47/-78, we generated progressive truncations of the ΔNp73 promoter and a deletion mutant lacking the p53 responsive element (**Fig.10A**). Luciferase constructs containing these truncated promoters were tested for the ability to respond to p53. Constructs containing more than 150 bases upstream of the TATA-box showed strong luciferase activity by p53 cotransfection (constructs A-D) while shorter constructs lacking the p53 responsive element were no longer inducible (constructs E and F) (**Fig.10B**). Similarly deletion of two of the p53 half-binding sites (constructs A.del and B.del) abrogated the induction by p53 completely demonstrating the existence of a single active p53 binding site (**Fig. 10B**).

In order to confirm that p53 and TAp73s were able regulate ΔNp73 we studied the expression levels of endogenous ΔNp73 after transfection of SAOS-2 cells with p53 or TAp73α and β. Transcript analysis by real time RT-PCR (**Table 2**) shows that p53 is capable of inducing both TA and ΔNp73 constructs, however while TA forms are induced 26 fold ΔNp73 is induced up to 3620 fold. Similarly TAp73 α and β are capable of inducing ΔNp73, β having the strongest activity, up to 5763 fold.

ΔNp73 blocks it's own induction by p53 and TAp73.

Finally, since ΔNp73 is capable of counteracting the activity of p53 and p73 we investigated the possibility that ΔNp73 blocked it's own induction. Co-transfection of p53 or TAp73α together with increasing concentrations of ΔNp73 and ΔN promoter construct shows that ΔNp73 is capable of blocking the action of p53 and TAp73 on it's own promoter (**Fig.11**), without transactivation activity on its own. This creates an additional feedback loop that finely tunes the system.

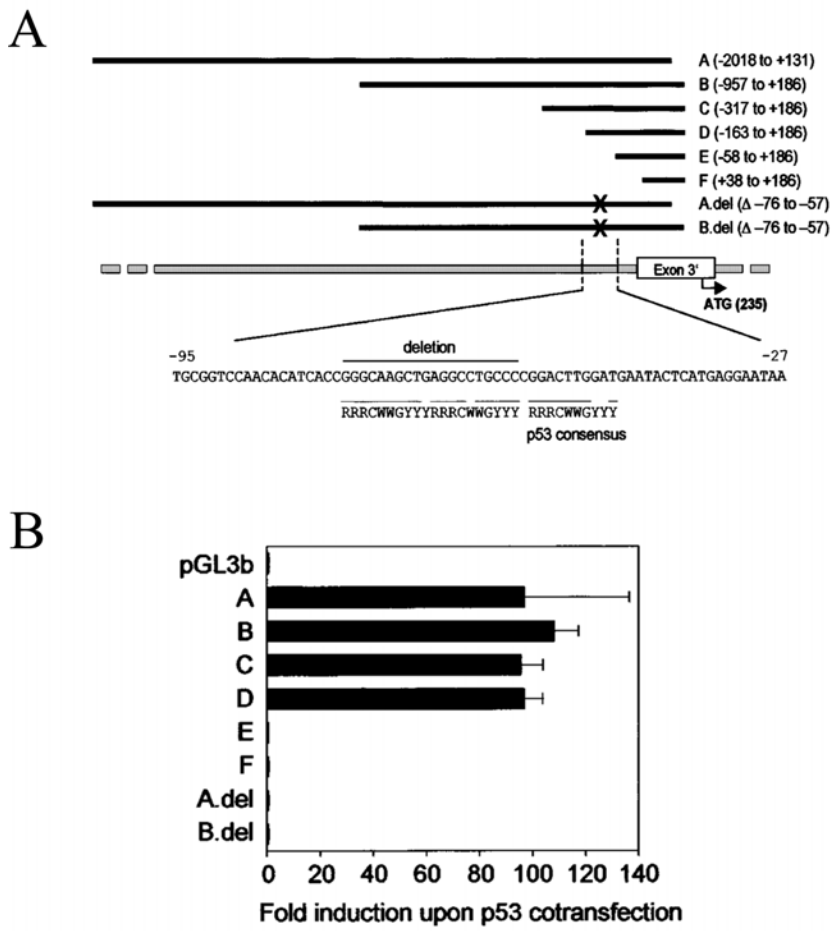


Figure 10. The ΔNp73 promoter contains a functional p53 responsive element.
(**A**) Schematic representation of genomic region upstream of exon 3' (open box) containing the ΔNp73 promoter. The sequence from bp-27 to bp-95 containing the p53 responsive element is shown. A to F progressive truncations of the ΔN promoter used for luciferase assays, the numbers in brackets indicate the nucleotides of origin and end of the fragments numbered from the cap site. A.del and B.del constructs contain a twenty base pair deletion. Deleted nucleotides are indicated. (**B**) Analysis of deletion mutants of ΔNp73 promoter (A to F and A.del and B.del) co-transfected with a p53 expressing plasmid by luciferase assay. Values are expressed in fold over control cells, transfected with an empty vector (pcDNA3) and the corresponding reporter constructs (A-F, A.del, B.del). Values are a mean of three experiments.

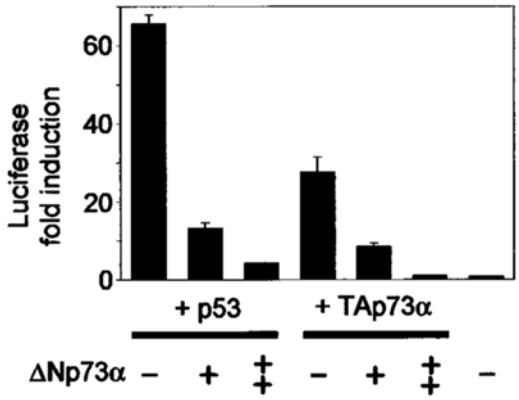


Figure 11. ΔNp73 protein blocks its own activation by p53 and TAp73.
Luciferase Assay in SAOS-2 cells. Cells were co-transfected with ΔNpromoter-Luciferase, p53 or TAp73α and increasing doses of ΔNp73 protein. ΔNp73 protein is not able to induce transactivation of it own promoter.

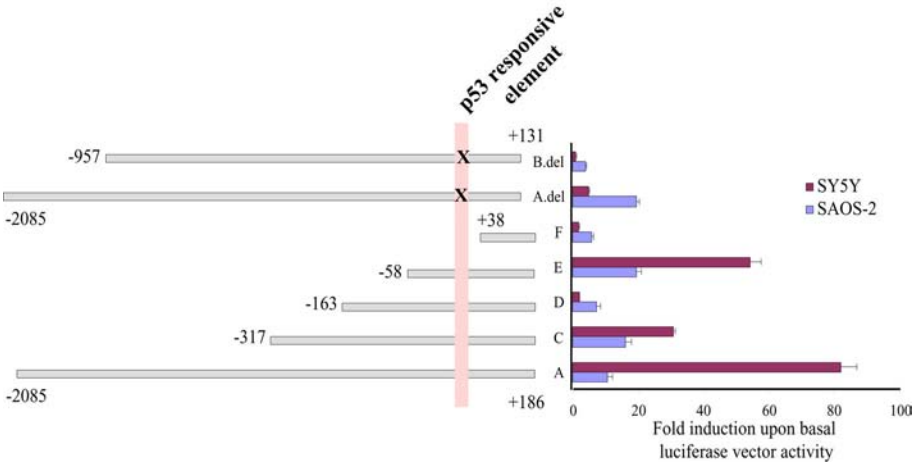


Figure 12. The ΔN promoter basal activity depends on the cell line.
SAOS-2 or SH-SY5Y cells were transfected with the different promoter deletions cloned in Luciferase vector. As previously, Luciferase activity was normalized by Renilla co-transfection.

Characterization of ΔN promoter responsive elements

In order to characterize other regulating regions in the ΔN promoter, we performed luciferase assays with the various deletions of the promoter, in different cell lines such as SAOS-2 and SH-SY5Y, a neuroblastome cell line (**Fig.12**). In this way, we were able to study the endogenous regulating factors effects and to determine the ΔN promoter regions involved in the differential expression of ΔNp73 according to the cell type. We first observed that p53 seems to be the major transcription factor which regulates ΔNp73 expression as shown by the lower promoter basal activation in SAOS-2, which is p53-deficient. Moreover, the p53 responsive element mutation completely abolish the basal activity in SY5Y cells. Secondly, it appears that possible other responsive elements may regulate the promoter activity, and may be present between -317 and –163 as suggested by the enhanced activity between the D and E constructs. It appears clearly from this experiment that the cell type is very important to study the promoter activity, as the basal regulations change from one cell line to the other.

Study of N-Myc activity on ΔNp73 promoter.

As it was known that human ΔNp73 up-regulation is often associated to bad prognosis neuroblastomas (Fillipovitch et al. 2001, Casciano et al. 2002a), a cancer type where N-Myc oncogene is also often amplified, we investigated if N-Myc was able to induce ΔNp73 transcription. Sequence analysis of the ΔNp73 promoter with responsive elements research software (TFSearch®) allowed to characterize several binding sites for different transcription factors (**Fig.13**). Several NF-κB and p300 binding sites were found between –1740 and -490, one E2F and one N-Myc at position –1800.

Luciferase assays were performed in 2 different cell lines : SK-N-AS, a neuroblastoma cell line which presents the 1q36 deletion and has therefore non detectable p73 levels; and SH-EP cells, which are N-Myc deficient. In this way, the basal activity level of ΔNp73 promoter is almost null and any activation by N-Myc over-expression can be detected. Numerous Luciferase assays were performed on ΔNp73 promoter, using p53 induction as a positive control. Although N-Myc was expressed (**Fig.14A**), no activation of the ΔNp73 promoter could be detected either in SK-N-AS cells, nor in SH-EP cells, even if N-Myc was correctly expressed (**Fig.14B**). Quantitative

RT-PCR performed in order to amplify specifically ΔNp73 transcripts were not able either to show any activation of ΔNp73 by N-Myc over-expression.

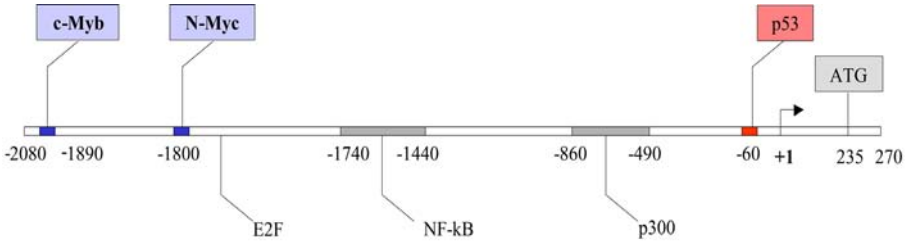


Figure 13. Characterization of different responsive elements present in ΔNp73 promoter.

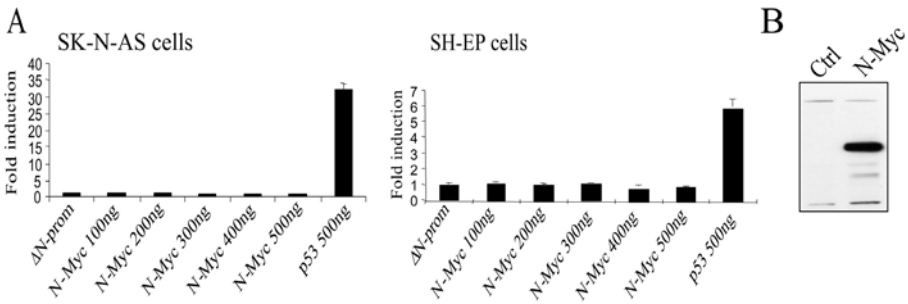


Figure 14. N-Myc over-expression doesn't induce ΔNp73 promoter activation.
(**A**) Luciferase activity assay performed in SK-N-AS or SH-EP cells. Cells were co-transfected with ΔNp73 promoter (1,5µg) and increasing doses of N-Myc expression vector or 500ng of p53 expression vector. (**B**) Western Blotting analysis of N-Myc over-expression in 293 cells. The blot is revealed by an anti-N-Myc antibody (Santa Cruz).

Generation of a ΔNp73α-inducible cell line

As we were not able to demonstrate any activation of N-Myc on ΔNp73 promoter and, moreover, as new statistical studies showed the lack of clear correlation between N-Myc amplification and ΔNp73 up-regulation, we decided to investigate the stability of the protein, in order to find a possible mechanism to explain its up-regulation in some neuroblastomas.

First of all, to work with a more physiological system, with a constant and controlled expression of ΔNp73 protein, we generated an inducible cell line in SAOS-2 cells. SAOS-2 cells stably transfected with the TET-on vector were transfected with HA-ΔNp73α cloned under the control of a TET Responsive Element. Several clones were selected by Hygromycin and chosen for their growth rate, ΔNp73 protein expression level and localization. One clone has been chosen for its ΔNp73 progressive expression after Doxycycline induction with a pick at 24 hours, as shown by western blot analysis (**Fig.15A**), using an anti-HA antibody. Moreover, ΔNp73 protein is only nuclear and doesn't induce apoptosis, on the contrary to TAp73α protein induced in SAOS-2 cells (**Fig.15B and C**). It is noteworthy that the SAOS-2(HA-ΔNp73α) cells tend to express the protein even in absence of Doxycycline induction, that is the "leakiness" of the clone. Hence, as HA-ΔNp73α can act as an oncogene, as we have seen before, these cells shouldn't be splited too many times to avoid the selection of "always-expressing" sub-clones.

HA-ΔNp73α decreases after UV treatment, in a dose and time dependent manner.

In order to further characterize the inducible cell line, we investigated the ΔNp73 anti-apoptotic role after DNA damage. Hence we irradiated cells with UV B and C (70 J/m^2) after 18 hours doxycycline treatment. Surprisingly, measurement of apoptosis by counting hypodiploid events didn't show any rescue of the ΔNp73-induced irradiated cells (**Fig.16A**). A Western Blot analysis of the same samples revealed that the HA-ΔNp73α levels dramatically decreased after UV treatment (**Fig.16B**). A dose response experiment showed that at low exposure levels to UV (35 J/m^2), ΔNp73 levels decrease but completely disappear at higher UV exposure levels (70 and 140 J/m2) 3 hours after irradiation (**Fig.16C and D**). Hence, HA-

ΔNp73α decreases after UV irradiation and this decrease is paralleled by the increase of apoptosis rate.

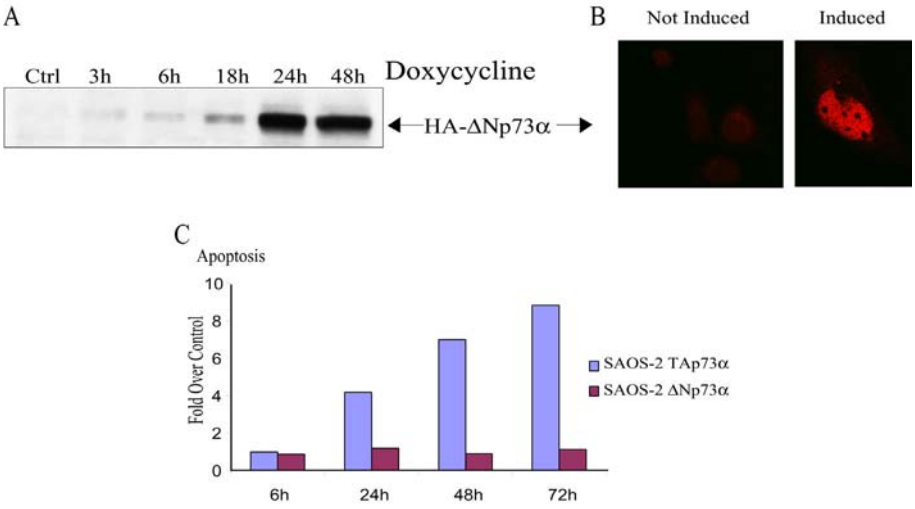


Figure 15. Characterization of the SAOS-2 (HA-ΔNp73α) inducible cell line.
(**A**) Western Blotting analysis of the HA-ΔNp73α expression after induction (3h to 48h) by Doxycycline (2μg/mL). The protein levels are revealed by an anti-HA antibody (BABCO). (**B**) Confocal microscopy analysis of the SAOS-2(HA-ΔNp73α) cells, induced or not by 24h Doxycycline treatment. The protein level is revealed by an anti-HA antibody (Santa-Cruz). (**C**) Evaluation of hypodiploïd apoptotic events after PI staining of SAOS-2 (HA-ΔNp73α) or (HA-TAp73α) inducible cells after 6 to 72h Doxycyclin treatment. The results are presented as Fold over the control rate of apoptotic events.

UV treatment induces specifically ΔNp73 degradation.

HA-ΔNp73α expression is controlled by the "reverse TET repressor", activated by Doxycycline, which binds the TET-Responsive Element, upstream the HA-ΔNp73α gene. In order to determine if the decrease observed was due to inhibition of transcription, for example by UV-induced Doxycycline degradation, we performed a semi-quantitative RT-PCR on SAOS-2(HA-ΔNp73α) cells and SAOS-2(HA-TAp73α) as a control (**Figure 17A**). The result clearly showed that the transcripts levels remains constant after UV irradiation in both ΔN and TA cell lines.

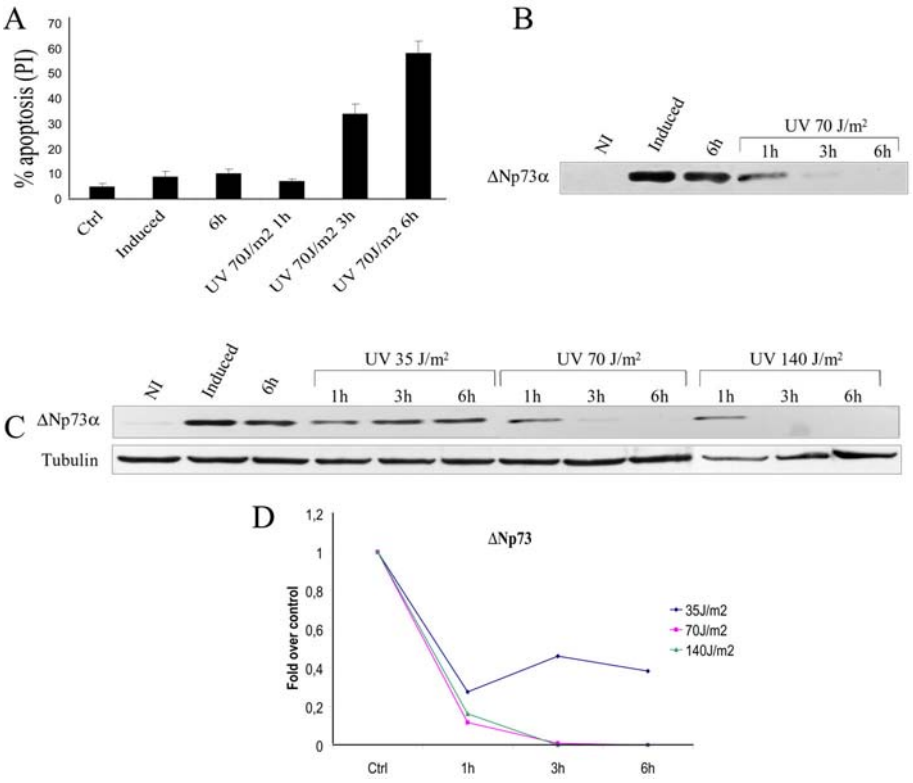


Figure 16. HA-ΔNp73α decreases after UV treatment, in a dose and time dependent manner.

(**A**) Evaluation of hypodiploïd apoptotic events after PI staining of the HA-ΔNp73α inducible cells. Cells were treated with Doxycycline for 18 hours. Doxycycline was then removed and cells were UV irradiated with 70 J/m^2 and harvested after 1, 3 or 6 hours. A non irradiated control is also collected after 18 hours (Induced) of induction and 6 hours after removing doxycycline (6h). (**B**) Western blot analysis of the same samples as (A), the HA-ΔNp73α levels were revealed by an anti-HA antibody (BABCO HA-11). (**C**) Western Blot analysis of the HA-ΔNp73α levels in the inducible cell line using the anti-HA antibody (BABCO HA-11). Cells were induced as previously and UV irradiated with 35, 70 or 140 J/m^2. They were harvest after 1, 3 or 6h. "Induced" is for the non irradiated control collected after the 18 hours induction. "6h" is collected 6h after removing Doxycycline, as a control for the decrease of Doxycycline induced HA-ΔNp73α transcription. (**D**) Densitometry analysis of the western blot shown in C demonstrating the time and dose dependency of the ΔNp73α degradation.

The presence of a transcript in the not induced sample may be explained either by the presence of small amount of gDNA which may contain more than one copy of the TET vector, or by the basal expression of the gene due to the Tet-on system which contains a minimal Cytomegalovirus promoter downstream the Tet responsive element that may be transcriptionaly active even without Doxycycline. The same UV treatment (140 J/m^2) was performed on SAOS-2(HA-TAp73α) and SAOS-2(p53), induced by doxycycline. Cells were harvested after 6 or 24 hours, the HA-TAp73α and p53 levels were revealed by anti-HA and anti-p53 antibodies. The protein levels remain constant after UV irradiation, indicating that ΔNp73α decrease may be due to a specific UV induced degradation of the protein (**Fig.17B**). Finally, we transfected SAOS-2 cells with GFP and non-tagged ΔNp73α and ΔNp73β. The protein levels were revealed by a polyclonal anti-p73 antibody (see characterization below) and showed a decrease after UV irradiation, indicating that the ΔNp73 degradation is independent of the C-terminal variant, that is, with or without the SAM domain, and of the N-terminal HA tag (**Fig.17C**).

ΔNp73 is degradated after DNA damaging agents treatments.

ΔNp73α degradation is not restricted to treatment with UV but is obtained with a number of different DNA damaging agents, as shown in **Figure 18**. Treatment of induced SAOS-2(ΔNp73α) with doxorubicin (1 and 3 μM) and etoposide (25 and 50μM) results in the disappearance of the ΔNp73α protein 24 hours after drugs treatment. Etoposide inhibits Topoisomerase II, the enzyme responsible for relaxing DNA helix during duplication by cutting the double strand, removing supercoils and ligate again double strand. Etoposide blocks the TopoisomeraseII-induced ligation thus generating double- and single-strands breaks. Doxorubicin (or Adramycin) intercalates with the DNA helix, thereby inhibiting duplication. It seems to inhibit also Topoisomerase II.

Thus, even if the pathways involved in UV-induced and drugs-induced DNA damage are different, it seems that they both involve ΔNp73α degradation.

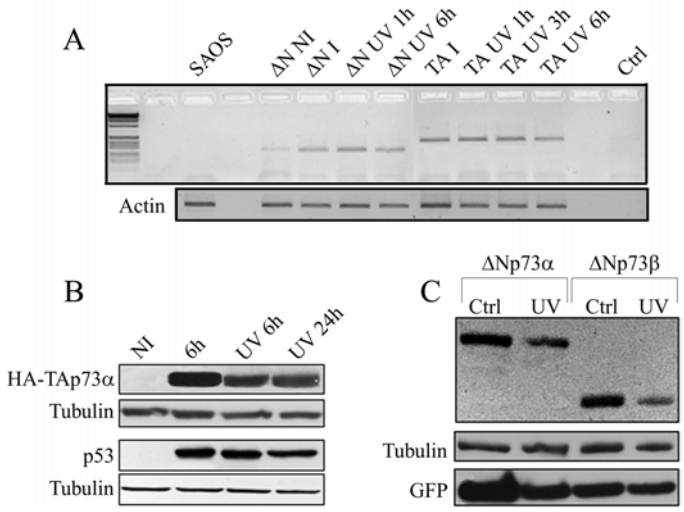


Figure 17. UV treatment induces specifically ΔNp73 degradation.
(**A**) RT-PCR for TAp73 and ΔNp73 transcripts using mRNA extracted from SAOS-2(HA-ΔNp73α) and SAOS-2(HA-TAp73α) induced (I) or not (NI) to express ΔNp73 or TAp73 respectively and then treated with UV (140 J/m^2) as described above and collected after 1, 3 and 6 hours. (**B**) Western blot analysis of SAOS-2(HA-TAp73α) and SAOS-2(p53) cells using anti-HA (BABCO, HA-11) and anti-p53 antibodies (Santa Cruz, DO-1 and 1801). Cells were induced or not (NI) as described above and treated with 140 J/m2 UV. They were harvested after 6 or 24h and a non irradiated control is also collected after 6 hours. (**C**) Western blot analysis of SAOS-2 cells transfected with plasmids expressing: green fluorescent protein GFP and not tagged ΔNp73α or ΔNp73β using Lipofectamine2000 (Invitrogen Life Technologies, Basel, Switzerland) according to the manufacturer's protocol. Protein extracts of Untreated (ctrl) or UV treated cells (UV) (35 J/m^2) were blotted and stained using a polyclonal anti-p73 antibody (See characterization).

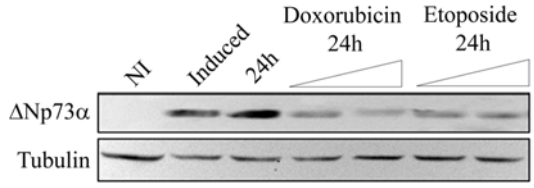


Figure 18. ΔNp73 degradation is induced by DNA damaging agents.
Western Blot analysis of the HA-ΔNp73α levels in the inducible cell line using the anti-HA antibody (BABCO HA-11). Cells were induced by a 18 hours Doxycyclin treatment. Doxycyclin was removed and cells were collected immediately (Induced) or 24h after Doxycyclin was removed. Cells were treated with Doxorubicin (1 and 3 μM) or Etoposide (25 and 50 μM) and harvest after 24h.

Endogenous ΔNp73 is degradated after UV irradiation, characterization of a polyclonal anti-p73 antibody.

At this point, we wanted to investigate the endogenous ΔNp73 degradation, but, as we have seen before, all the endogenous p73 isoforms are usually expressed at very low level and are hard to detect by western blot analysis. As the first antibody we used (Fig3B and 7, provided by D. Caput) was not available any more and was α isoform specific, we decided to generate a new one. A rabbit was immunized with a p73 C-terminal-domain peptide, in order to obtain an antibody which could recognize all the p73 isoforms. As the anti-serum gave clean immunostaining, we decided to use it without further purification., thus "anti-p73 antibody" will be in fact for "anti-serum".

The western blot analysis of over-expressed p73 isoforms revealed first that the new anti-p73 antibody doesn't cross-react with HA-TAp63α nor HA-ΔNp63α, and neither with p53 (**Fig.19A**). Secondly, we observed that, even if the antibody is supposed to recognize all the p73 isoforms, the anti-p73 binds much more strongly the α forms (TA and ΔN), indicating that the peptide synthesized may present more epitopes of the α than the other isoforms. However, compared to the 2 commercial antibodies (Neomarker) Ab2, which recognizes the α forms (**Fig.19B**), and Ab4, which recognizes the α and β forms (**Fig.19C**), our antibody gives a stronger signal for all these isoforms. Finally, the new anti-p73 antibody gives good signal of fluorescent immuno-staining in the inducible cells (**Fig19D**).

Figure 20 shows that our antibody is able to immunoprecipitate the over-expressed HA-TAp73α, in a dose-dependent manner, and at a level comparable to the Ab4 (Neomarker) immunoprecipitation.

To test the new anti-p73 antibody ability to detect the p73 endogenous levels, we performed western blot analysis on SK-N-SH (a human neuroblastoma cell line) and HaCaT (human keratinocytes) cells, as they present good levels of p73 transcripts and proteins (**Fig.3B and Table1**). As it has been shown that p73α levels increase in neuroblastoma induced to differentiate by Retinoic Acid (De Laurenzi et al. 2000), we treated SK-N-SH cells with RA for 5 days, and performed a western blot analysis of the nuclei extracts. We observed a small increase of the protein (**Fig.21A**), but the levels remained very low. On the contrary, the proliferating HaCaT cells showed a high level of p73 expression (**Fig.21A**).

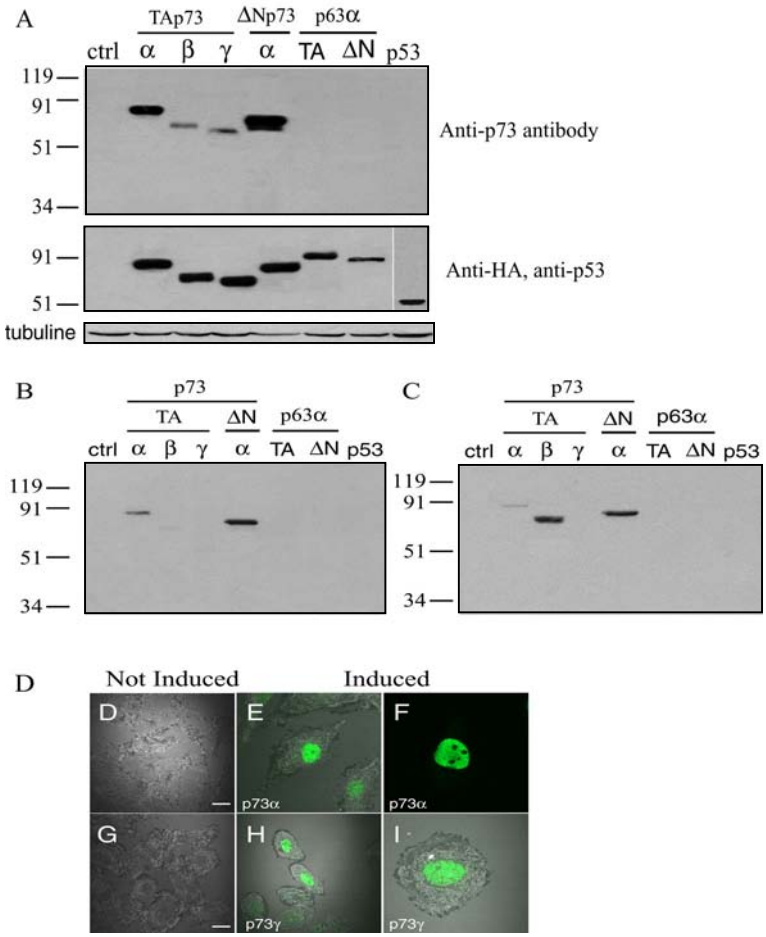


Figure 19. Our p73 antibody is specific for all p73 isoforms, but doesn't cross-react with p53 and p63 isoforms.

(**A**) Western blot analysis of SAOS-2(p53 family members) inducible cell lines, using our p73 rabbit antibody (upper) and anti-HA (Santa Cruz Y-11) and anti-p53 (Santa Cruz DO-1) antibodies (middle), as a control of protein expression. (**B**,**C**) western blot analysis using commercial antibodies for p73. p53 family members expression analysis using the Neomarkers Ab2 (panel B) and Ab4 (panel C) clones. (**D**) SAOS-2(HA-TAp73α) and (HA-TAp73γ) were analysed by confocal microscopy, using our anti-p73 (panels D, E, H) and anti-HA antibodies (F, G, I). Both antibodies recognize the same nuclear protein. No signal was detected in not induced cells (D,G).

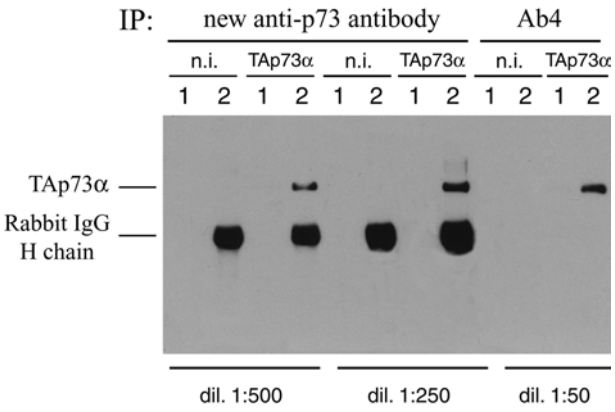


Figure 20. p73 immunoprecipitation.
TAp73α inducible cells were lysed and proteins were immunoprecipitated using our polyclonal anti-p73 antibody or a commercial monoclonal one (clone Ab4, Neomarkers). Proteins were detected with rabbit anti-HA antibody. The polyclonal anti-p73 antibody is able to immunoprecipitate TAp73α in a dose-dependent manner, like anti-p73 Ab4 antibody. (n.i.: not induced cells).

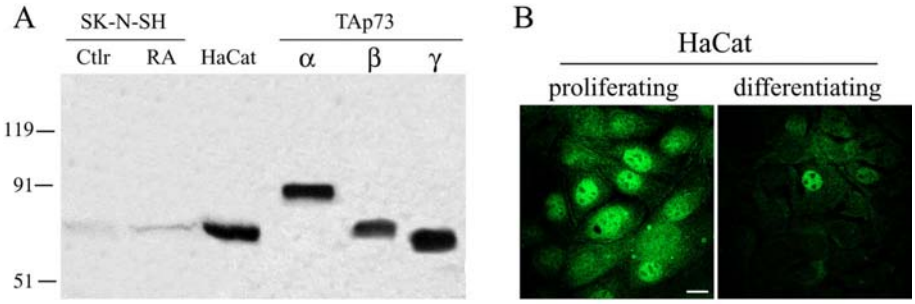


Figure 21. p73 endogenous level.
(**A**) Western blot analysis of endogenous p73 expression in SK-N-SH and HaCat purified nuclei, using polyclonal anti-p73 antibody. SK-N-SH cells were treated with retinoic acid (5μM, 5days) to induce differentiation, while HaCat cells were grown in normal, proliferating conditions. The SAOS-2(HA-TAp73α,β) and (HA-ΔNp73α) cell lysates were used as positive controls. (**B**) Confocal microscopy analysis of the HaCaT cells grown in proliferating or differentiating (1,2mM Ca^{++}, 5 days). Confocal microscopy analysis revealed a strong diminution of ΔNp73α expression in differentiating HaCat cells.

The 3 positive controls (TA and ΔNp73α and TAp73β) on the same western blot emphasize the difficulty to determine the endogenous isoforms, as they separate very closely. The p73 isoform detected in HaCaT should be the ΔNp73α isoform (cf. Fig.3B and Table1), but it is clear that it remains difficult to characterize. **Figure 21B** tends to confirm this, as the protein stained in HaCaT cells disappears after differentiation, as expected from the anti-apoptotic role of ΔNp73 and from the ΔNp63 expression pattern in epithelium (See Introduction Fig.31). Therefore, to characterize the endogenous p73 isoforms, one should always load all the p73 positive controls.

We studied endogenous ΔNp73 degradation in the H1299 cells, a p53-deficient lung carcinoma cell line, which present a good level of p73 protein. Cells were plated the day before, UV irradiated (140 J/m^2) in PBS and replaced in fresh medium for 6 or 24h. Nuclei were purified and lysed, in order to increase the p73 protein concentration in the lysate. Western blotting analysis using our polyclonal anti-p73 antibody revealed that the endogenous level of ΔNp73γ decreases after UV irradiation (**Fig.22A**). Moreover, the FACs analysis of the hypodiploïd events in the same samples showed a clear correlation between ΔNp73γ degradation and the apoptosis increase (**Fig.22B**)

ΔNp73 degradation occurs in a proteasome-dependent manner.

MG132 is a potent, reversible, peptide aldehyde protease inhibitor, which is able to reduce the degradation of ubiquitin-conjugated proteins by the 26S proteasome complex (that is the 20S core subunit plus the 19S regulator subunit). 2 hours MG132 pre-treated SAOS-2(HA-ΔNp73α) cells were irradiated as previously described, harvested and lysed 6 hours after UV treatment. The western blot analysis showed a complete inhibition of ΔNp73 degradation in MG132 treated cells (**Fig.23A**), indicating that it may occur in a proteasome-dependent manner. Moreover, the MG132 induces a small rescue of cells, as shown in the apoptosis analysis (**Fig.23B**). This rescue is likely to be due to the high level of ΔNp73α, but may also implicate the degradation block of other unknown factors.

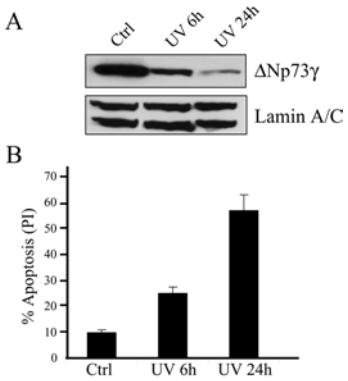


Figure 22. Endogenous ΔNp73 degradation in H1299 cells.
(**A**) Western Blotting analysis of endogenous p73 levels by a polyclonal anti-p73 antibody (see characterization). H1299 cells were plated at 20000/cm^2 the day before treatment. Cells were UV irradiated (140J/m^2) and harvest 6 or 24 hours after. Nuclei were purified, lysed and analysed by Western Blotting. (**B**) Evaluation of hypodiploid apoptotic events after PI staining of part of the cells harvested for the Western Blotting analysis.

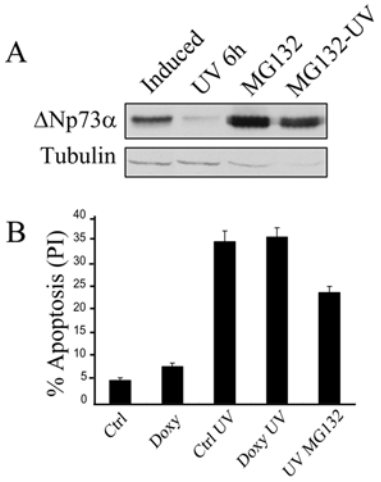


Figure 23. ΔNp73 is degradated in a proteasome dependent pathway.
(**A**) Western Blot analysis of HA-ΔNp73α levels using an anti-HA antibody (BABCO HA-11). HA-ΔNp73α expression was induced as previously described. Doxycycline was then removed, cells were pre-incubated 2h with the proteasome inhibitors MG132 (10μM), then treated with UV (140 J/m^2) and harvested after 6 hours. (**B**) Evaluation of hypodiploïd apoptotic events after PI staining of the HA-ΔNp73α inducible cells. Cells were induced by a 18 hours Doxycyclin treatment and then treated as for the western blot analysis. A not-induced control (Ctrl) was also collected.

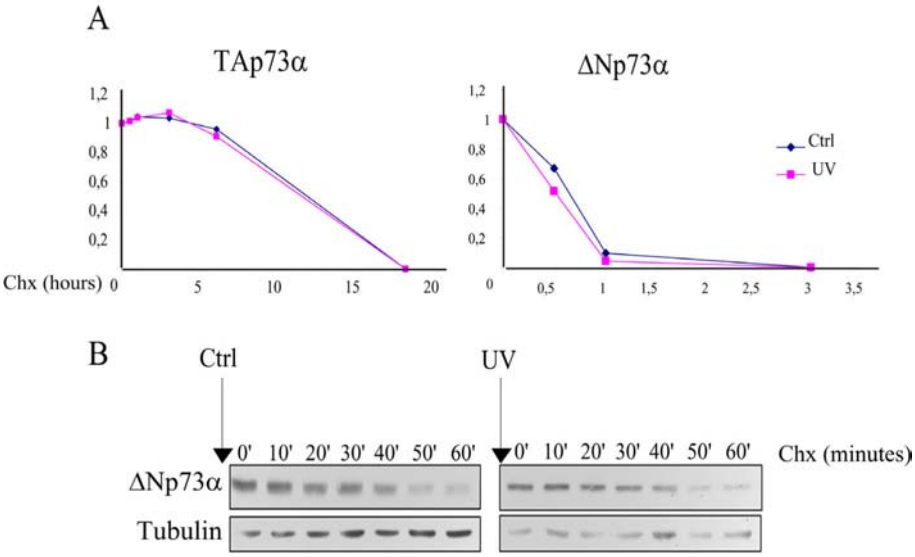


Figure 24. TA and ΔNp73 have got very different half-lives.
(**A**) Densitometric analysis of western blots for HA-ΔNp73 and HA-TAp73. Cells were induced by a 18 hours doxycyclin treatment. Doxycyclin was then removed and replaced by PBS. Part of the cells were UV irradiated (140J/m^2, equivalent to 30 seconds) and other were just left 30 seconds in PBS. PBS was removed and cells were placed in fresh medium containing 20μg/mL cycloheximide and harvested at different time points. Western blot analysis of HA-ΔNp73 and HA-TAp73 was performed as described above, densitometry analysis was normalized by tubulin levels. (**B**) Western Blot analysis of HA-ΔNp73 levels using an anti-HA antibody (BABCO HA-11). Experiment was performed as described in A and cells were harvested every 10 minutes.

It is noteworthy that the not-irradiated point, pre-treated with MG132 ("MG132"), presents ΔNp73α levels higher than the control point ("Induced"). This suggests a rapid turn-over of the protein, so we decided to study ΔNp73α degradation in absence of proteins production, in order to investigate the protein half-life changes after UV-irradiation.

TAp73α and ΔNp73α have very different half-lives

To evaluate a protein half-life, the current technique is the pulse-chase which indicates precisely the time necessary for a pool of new synthesised proteins to be degradated. However, cycloheximide (or Acti-Dione) blocks peptide synthesis, and thus gives a good indication of the protein half-life.

Figure 24A presents the densitometric analysis of a western blot performed on SAOS-2(HA-TAp73α) and (HA-ΔNp73α). It clearly appears that TAp73α completely disappears after 18 hours, but it take 1 hour to ΔNp73α to be completely degradated. Surprisingly, the ΔNp73α half-life is not affected by UV treatment, as TAp73α, used as a control. Therefore, we focused on the first hour after SAOS-2(HA-ΔNp73α) UV treatment, in order to study shorter periods. The western blot analysis (**Fig.24B**) didn't bring to the fore any differences between control and irradiated cells.

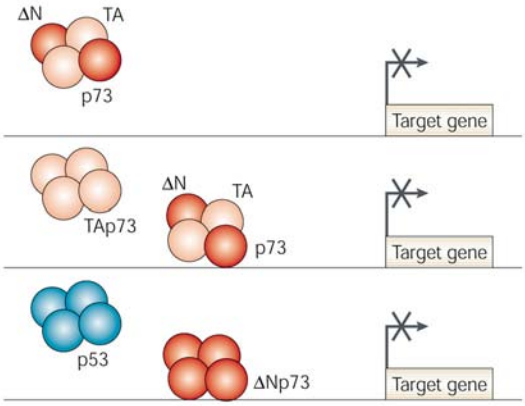
DISCUSSION & CONCLUSION

As we have seen before, in contrast to p53, p73 is expressed as several distinct forms differing either at the C- or at the N-terminus. Indeed, differential splicing of the 3' end of the gene leads to the expression of several p73 C-terminal splice variants (α to ζ) (Kaghad et al. 1997, De Laurenzi et al. 2000, De Laurenzi and Melino 2000, Scaruffi et al. 2000) that differ *in vitro* in their potential to activate p53-responsive genes, such as $p21^{Waf1/Cip1}$ and Bax, thus showing different functional properties. In addition, in mice two variants with different N-terminus have been identified, thought to derive from the usage of two different promoters one located upstream of exon 1 and one located upstream of exon 3' in the third intron of the gene (Yang et al. 2000). Consequently, full length isoforms contain the transactivation domain (TA forms) and shorter (ΔN) isoform lack this domain, introducing additional complexity to the system. The ΔNp73 variants do not activate transcription from p53-responsive promoters and inhibit the full length p73 variant (TAp73) probably acting as dominant negatives. Ectopic expression of these variants in mice was shown to inhibit p53-induced apoptosis and to protect $p73^{-/-}$ neurons from death induced by nerve growth factor (NGF) withdrawal (Pozniak et al. 2000).

When this work was completed (2001), it was the first report of cloning of the human ΔNp73 isoforms, previously described in the murine system (Yang et al. 2000, Pozniak et al. 2000). Our results showed that human ΔNp73 isoforms are highly homolog to the mouse ones, and that their expression is driven by a second promoter located in a genomic region upstream of exon 3'. The transcript presents two different ATG, which are both functional and used physiologically. ΔNp73 protein, as TAp73, is nuclear and expressed in different cell lines and tissues. However, in contrast to the murine embryo, TA forms are always expressed 10 to a 100 fold higher than ΔN isoforms in human cell lines or tissues we examined. This may be due to a different time and place expression of ΔNp73 protein in human embryonic development compared to the murine one. In addition, most of the tumor cell lines tested showed an altered TA/ΔN ratio when compared to normal adult or fetal tissues, suggesting that the relative amounts of the two forms rather than their absolute amount is important for normal cell function.

In fact, the net activity of the TA forms is tightly regulated by the dominant negative effect of ΔN present in the cell. In addition ΔN isoforms can interact with p53 and regulate its function. We showed in this study that ΔNp73 is able to counteract TAp73 and p53 transcriptional activity on target genes, such as p21 and Bax, and therefore to inhibit their pro-apoptotic or

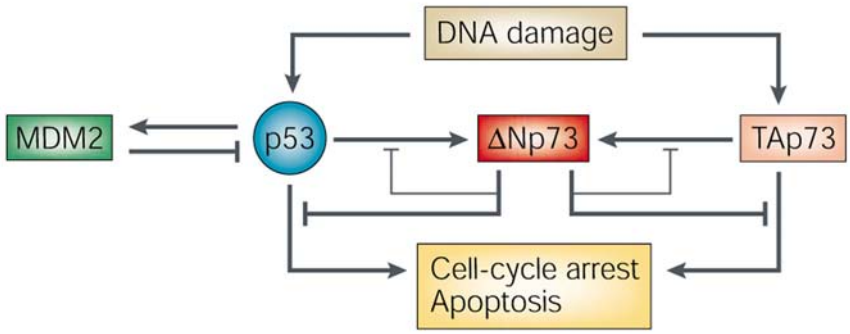
cell cycle arrest action. Experiments with ΔNp73 DNA binding mutants let us hypothesize that ΔNp73 may form tetramers which compete with p53 responsive-elements on the target genes promoters, while TAp73 may be sequestered by forming inactive tetramers with ΔNp73 proteins, as presented below.



We characterized ΔNp73 promoter, localized in the TAp73 third intron, and, very interestingly, we found a p53 responsive element situated 65bp upstream the initiation of transcription site. As p73 DNA binding domain is highly homolog to the p53 one, TAp73 is likely to bind this responsive element and activate also ΔNp73 transcription. Thus, our results showed that p53 and TAp73 are capable of inducing ΔNp73 which in turn blocks p53 and TAp73 action by competing for DNA binding or sequestering the protein. In addition ΔNp73 counteracts p53 and TAp73 action on its own promoter creating an additional feedback loop that allows for the fine tuning of the entire system. p53 is expressed in response to DNA damage and leads to cell cycle arrest and apoptosis (Levine 1998). As for other killing genes, p53 must be carefully regulated to avoid wanton cell death. Intracellular p53 levels are therefore controlled by a weighted balance of production and degradation. High levels of p53 determine its own regulation by inducing the transcription of MDM2 that binds p53 and induces its ubiquitination and degradation in proteasome (Lohrum and Vousden 1999).

Here we described a novel way of controlling p53. We also showed that a similar loop regulates TAp73, since ΔNp73 is induced by TAp73, and

ΔNp73 also regulates the function of the TA forms, which is stabilized after DNA damage (see below).



Human ΔNp73 regulates a negative feed-back loop for TAp73 and p53.

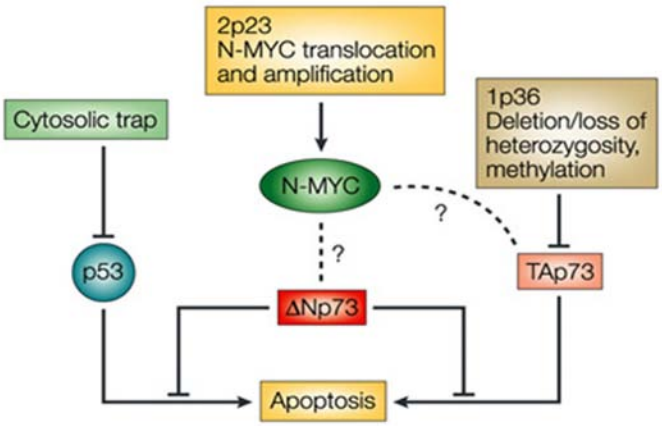
The major implication of these results is that ΔNp73 plays an important role in regulating p53 function and that its over expression could play a role in tumorigenesis by generating a functional block of p53. The net functional result on a cancer cell would mirror the effect of the "classical" structural inactivation of the p53 gene through allelic deletion and inactivating mutations. Complete absence of p73 would inflict less damage to most cells than the altered equilibrium of its isoforms, explaining the absence of tumors in $p73^{-/-}$ animals.

A very interesting additional observation of ours is the relatively high expressions levels of p73 in cancer cell lines. Indeed, we should remind that several cancers express a cancer-specific TA isoform of p73 with a specific deletion of exon 2 (Δ2-p73) that, because of the disruption of the TA domain, mirrors the function of ΔNp73 (Casciano et al. 1999, Fillipovitch et al. 2001). It is possible that increased ΔN levels lead to a functional inactivation of p53 and thus become involved in tumorigenesis.

As we have seen, the p73 gene maps to a region (1p36.33) that is frequently deleted in neuroblastoma, indicating that it might have a role in the development of this tumor. However, p73 is rarely mutated in neuroblastoma which implies that p73 is not a classical tumor-suppressor gene. Intriguingly, in neuroblastoma, p53 is also rarely mutated but trapped in the cytosol in neuroblastoma, which inhibits its transcriptional activity.

Overexpression of the ΔNp73 isoform could generate a functional block of p53. The net result on a cancer cell would mirror the effect of structural inactivation of p53, and a functionally p53-negative, aggressive tumor would result. Methylation in the p73 promoter has been described (Casciano et al. 2002b), indicating a possible mechanism for the differential regulation of TAp73 and ΔNp73.

Two biological and prognostic important genetic events are crucial in neuroblastoma, occurring in 2p23 (harboring N-Myc) and 1p36 (harboring p73) (Brodeur et al. 1995). In fact, both N-Myc functional amplification and ΔNp73-isoform expression are strong prognostic factors (Casciano et al. 2002a). Whereas c-Myc seems to inhibit TAp73 transcriptional activity (Watanabe et al. 2002), this is not generally accepted (Zaika et al. 2001), and no data are available on N-Myc and ΔNp73 (see model below). In fact, in our experimental conditions, we couldn't detect any activation of ΔNp73 transcription, either on SK-N-AS or on SH-EP cells.



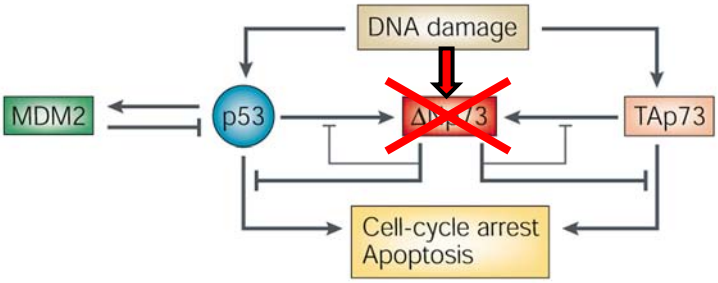
Casciano et al. in 2002 showed that expression of the ΔNp73 isoform, and not of TAp73, correlates with both poor overall and progression-free survival in neuroblastoma patients. ΔNp73 is strongly associated with reduced survival and predicts a poorer outcome, independent of age, primary tumor site, stage and N-Myc amplification.

However, studying the ΔNp73 promoter sequence, we found several interesting responsive elements, which may be involved in ΔNp73 transcriptional up-regulation, either during physiological processes (such as

CNS neuronal development and maintenance in mice), or during pathology development (for example in neuroblastoma). It is noteworthy that the basal activity of the promoter and its different deletions was extremely different in diverse cell lines, suggesting the presence of other binding sites, even if the major activation seems to be due to p53 protein. For example, the presence of 11 NFκB binding sites, in a 300bp region, may play a role in cell survival and proliferation. Moreover, the NFκB binding sites are often retrovirus targets for integration in the cell genome, which may explain the generation of this alternative, new promoter appeared in the p63 gene, after the vertebrates transition (see Fig.25 Introduction).

Hence, the ratio between TA/ΔN isoforms seems to be the major point for physiological conditions. As p53, TAp73 protein is finely regulated by post-translational modifications in order to respond to stresses or differentiating signals. The modifications which may induce ΔNp73 stabilization or degradation are still poorly understood.

Our data show that, after UV irradiation or drug-induced DNA damage, over-expressed ΔNp73 protein is rapidly degraded, probably in a proteasome dependent manner. Moreover, the new anti-p73 antibody we generated allowed us to describe this phenomenon also at the endogenous level.



DNA damage induces the rapid and selective degradation of the ΔNp73 isoform, allowing apoptosis to occur.

Since ΔNp73 levels can be controlled by TAp73 and p53, the existence of ΔNp73 represents a safety system preventing wanton cell death. However this direct control implies that ΔNp73 levels increase in response to DNA damage (Vossio et al. 2002) (in a p53/TAp73 dependent manner) preventing

p53 and TAp73 from exerting their action on damaged cells. It is therefore necessary that this safety block is removed when cells are irreversibly damaged. We show for the first time that ΔNp73 is rapidly degraded upon DNA damage while p53 and TAp73 proteins are not. As a consequence, upon DNA damage ΔNp73 levels initially rise and then decrease in a dose dependent manner while TAp73 and p53 levels are increased and remain high. These results may be correlated to the study of p63 transgenic mice (Liefer et al. 2000) which showed that p63 down-regulation is required for epidermal UV-B induced apoptosis. Moreover, in "resting" human keratinocytes, ΔNp63 is predominant, thus potentially blocking p53 function. Following UV radiation, ΔNp63 is lost by degradation and p53 levels enhanced by stabilization in response to DNA damage (McKeon 2002).

How this degradation occurs or is induced is still unknown. As we performed the experiments in p53-null cells, this process should not involve a p53 dependent pathway. However, it is of interest that after DNA damage TAp73 is stabilized and activated, while the ΔN isoform is rapidly degraded, indicating that these two isotypes are under independent controls. The study of TA and ΔN N-terminal domains, may allow to find different post-translational modifications or regulators binding sites. For example, Mdm2 binds to the TAp73 N-terminal domain without targeting it for degradation, but it is unknown what kind of modification Mdm2 may induce on ΔNp73 protein, if it does. Here is reported the TA (blue) and ΔN (red) N-terminal domains, with potential phosphorylation sites underlined in yellow.

Our data also show that ΔNp73 protein has a very short half life (about 20minutes) if compared to the TAp73 isoform (18hours) suggesting that ΔNp73 protein levels are normally kept very low in cells. It would be also very interesting to understand which regulators may differentially control the TA and ΔN stability and degradation in physiological conditions. Even if it is clear that TA and ΔN are degraded in a proteasome dependent manner, very little is known about the precise mechanism by which it occurs and it is regulated. However, as ΔNp73 has been shown to have transforming activity (Stiewe et al. 2002; Petrenko et al. 2003) and high levels of ΔNp73 have

been associated with a number of tumors (Zaika et al. 2002) and with poor prognosis (Casciano et al.2002a), it is very important for ΔNp73 to be kept at very low level in the cell, with a very rapid turn-over. An excessive ΔNp73 stability after DNA damage or in physiological conditions may induce cell transformation, as it may occur in neuroblastoma.

Conclusions

Even if the p53 stability and degradation pathway has been well studied, the mechanisms elucidated for p53 are rarely applicable to p63 or p73. It is clear that p73 and p63 stability is regulated not only by protein modifications (phosphorylation, acetylation) as in the case of p53, but also by the relative amounts of the individual splicing variants, thus at the level of the splicing machinery, which implies yet unknown regulators. Moreover, as we have seen in the introduction, p63 contains an Inhibitory Domain downstream of the SAM domain, which implies, via interactions between the different splicing variants, another level of regulation. As p63 and p73 present a high homology, p73 is likely to present the same Inhibitory Domain.

Moreover, p73 (and p63) present the ΔN isoforms, which play an important anti-apoptotic role, as they can inhibit p53 and TA isoforms activity, and be in turn transcriptionaly activated by them. Post-translational modifications which lead to ΔNp73 degradation in response to DNA damage are still unknown and add further complexity to the p73 proteins regulation.

This much higher complexity may be explained by the fact that p73 and p63 are phylogenetically older than p53. They are not just involved in apoptosis but also in neuronal or epidermal development, which requires a high capacity to respond to diverse stimuli in space and time during embryogenesis, and a fine equilibrium between differentiating and anti-apoptotic signals. p53 can be seen as a simpler form of these two proteins, ‘‘specialized’’ in inducing cell death, so understanding p63 and p73 stability regulation cannot be achieved by drawing simple analogies to p53 pathways. The identification of novel pathways of p63 or p73 stability makes the puzzle more complicated and shows that a lot remains to be understood, in particular, about the degradation pathways of p73 and p63 TA- and ΔN-forms.

MATERIALS & METHODS

Human ΔNp73 regulates a dominant negative feedback loop for TAp73 and p53 (Fig.1 to 14).

Cell cultures and samples.

Almost all cells were grown in Dulbecco's Modified essential Medium (DMEM) or RPMI-1640 supplemented with 10 % v/v Foetal Bovine Serum, 1,2 g bicarbonate per litre, 1% (v/v) non essential amino acids and 15mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in air. SH-SY5Y, SAOS-2, SK-N-SH and SH-EP cells were grown in a mix of MEM (Minimal Essential Medium) and Ham's F12 (1:1, w:w), supplemented with 15mM Hepes, 1,2g/L Sodium Bicarbonate, and 10% (v:v) of fetal bovine serum. All chemicals and reagents were from Sigma (Buchs, Switzerland), unless specified. Cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany) and from the American Type Culture Collection (Rockville, USA). The HaCaT cell line was a kind gift from N. Fusenig (Heidelberg, Germany). The panel of normal RNA was obtained from Stratagene (Amsterdam, The Netherlands).

Analysis of apoptosis.

To estimate DNA fragmentation, SAOS-2 cells were plated to approx. 50% confluency and transfected, using Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's protocol, with either TAp73α or p53 in combination with pcDNA3 or ΔNp73α, together with a GFP-spectrin expression vector at a 1 to 5 ratio. Cells were collected at 800 x g for 10 minutes and fixed with 1:1 PBS and methanol-acetone (4:1 v/v) solution at -20°C. Hypodiploid events and cell cycle of GFP positive cells was evaluated by flow cytometry using a propidium iodide (PI) staining (40 mg/ml) in the presence of 13 kU/ml ribonucleaseA (20 minutes incubation at 37°C) on a FACS-Calibur flow cytometer (Becton-Dickinson, CA, USA).

Cells were excited at 488 nm using a 15mW Argon laser, and the fluorescence was monitored at 578 nm at a rate of 150-300 events/second. Ten thousand events were evaluated using the Cell Quest Program (ibid). Electronic gating FSC-a/vs/FSC-h was used, when appropriate, to eliminate cell aggregates.

Fluorescent microscopy

SAOS-2 cells were transfected with TAp73α-GFP and ΔNp73α-GFP expression vectors with Lipofectamine 2000 (Life Technologies, Basel, Switzerland) according to the manufacturer's protocol. 36 hours after transfection, cells were fixed in 4 % paraformaldehyde. Slides were analysed with a fluorescence microscope (excitation wavelenght 490 nm) and images were aquired with a CCD camera (Biorad).

RNA extraction and reverse transcription.

Total RNA was extracted using the RNeasy kit (Qiagen, Basel, Switzerland). During the extraction procedure, contaminating DNA was removed by DNaseI: 600ng RNA was treated with 10U of DNaseI (Roche, Basel Switzerland) in a total volume of 10 µl containing 50mM NaAc pH 5,0, 5 mM $MgCl_2$ and 25U of RNase Inhibitor (Roche) for 15 minutes at 37°C, 5 minutes at 90°C and immediate placement on ice. 200ng of total RNA was reverse transcribed in a 20µl reaction volume, using 16nM pd(N)6 random primers, 19,2 U of avian myeloblastosis virus reverse transcriptase, 50U RNase inhibitor and 1x AMV-RT-buffer (all reagents from Roche). The incubation was at room temperature for 10 minutes, followed by 42°C for 60 min. and 95°C for 5 min. To check for contaminating genomic DNA, RNA samples were processed identically as for cDNA synthesis except that the reverse transcriptase was replaced by the same volume of water.

Real-time quantitative RT-PCR.

Real-time PCR was used for absolute quantitation of p73 N-terminal variants, 7S RNA was used for internal standard. Each PCR was carried out in a total volume of 25µl containing cDNA reverse-transcribed from 25ng and 0,375ng total RNA for p73 and 7S respectively. Dual labelled (FAM/TAMRA) gene specific probes and TaqMan Universal PCR Master Mix (Applied BioSystems, Rotkreuz, Switzerland) were used for the PCR. ΔNp73 was amplified using 5‘-GGAGATGGGAAAAGCGAAAAT-3‘ as the forward primer, 5‘-CTCTCCCGCTCGGTCCAC-3‘ as the reverse primer (both 300µM), and a ΔNp73 probe 5‘-CAAACGGCCCGC ATGTTCCC-3‘ (150µM). For TAp73, the primers were forward 5‘-GCACCACGTTTGAGCACCTC-3‘, reverse 5‘-TAATGAGGTGGTGGGC GGA-3‘ (both 300µM), and the probe was 5‘-TTCGACCTTCCC CAGTCAAGCCG-3‘ (150µM). For 7S RNA the primers and probe were: forward 5’-ACCACCAGGTTGCCTAAGGA-3’, reverse 5’-CACGGGA GTTTTGACCTGCT-3’, probe 5’-TGAACCGGCCCAGGTCGGAAAC-3’ (300µM each). All measurements were performed twice, and the arithmetic mean was used for further calculations. For the determination of absolute transcript number analysis, cDNA was amplified from JVM-2 cells (TAp73 F 5’-ACGCAGCGAAACCGGGGCCCG-3’, R 5’-GCCGCGCGGCTGCT CATCTGG-3’, ΔNp73 F 5’-CCCGGACTTGGATGAATACT-3’, R 5’-GCCGCGCGGC TGCTCATCTGG-3’) and from a 7S-plasmid with F 5’-GCTACTCGGGAGGCTGAGAC-3’, R 5’-AGGCGCGATCCCACTA CTGA-3’). The amplicons were cloned into the pcDNA3.1/V5-His vector as described and the constructs were verified by sequencing. After digestion with Nsi I (Roche), a T7-dependent RNA synthesis was performed with the RiboMAX™ Large Scale RNA Production System (Promega, Wallisellen, Switzerland) according to the manufacturer’s protocol. The synthesised RNA was extracted with a 5’-biotinylated oligo (5’-TTTCC ACACCCTAACTGACA-3’) and the mRNA Isolation Kit (Roche) and quantified spectrophotometrically. Molecular concentrations were calculated and random-primed cDNA synthesis was performed with the purified RNA

adjusted to 200ng with yeast RNA. A series of dilutions was prepared and measured by real-time quantitative RT-PCR as described above.

Determination of C-terminal p73 mRNA splice variants.

100ng of cDNA obtained as described above from 3 different cell lines (JVM-2, HaCaT, and MCF-7) were amplified by PCR with forward primers specific for TAp73 and ΔNp73 and a reverse primer common to both variants: TAp73 F 5'-AAGATGGCCCAGTCCACCGCCACCTCCCCT-3'(exon 2); ΔNp73 F 5'-ATGCTGTACGTCGGTGACCC-3' (exon 3'); common reverse primer R 5'-TCAGTGGATCTCGGCCTCC-3' (exon 14). The Expand High Fidelity PCR System enzyme was used as described above, but with an annealing temperature of 61°C. 0.1μl of the first PCR product was used as a template for a nested PCR using a forward primer in exon 8 and a reverse primer in exon 14, 15 cycles were used for TAp73 and 18 cycles for ΔNp73. A p73 cDNA probe was used for the sensitive and specific detection of p73 splice variants. The probe was amplified from the plasmid HA-p73α(pcDNA3) using the following primers: p73 probe-1015F, 59-GAAAAGCTGATGAGGACCACTAC-39; p73 probe-1232R, 59-GCTCTCTTTCAGCTTCATCAGG-39. The probe was DIG-labelled with the DIG DNA Labelling and Detection Kit (Roche) according to the manufacturer's specifications. Briefly, for each sample, 2μl of PCR product were separated on a 3% agarose gel, blotted onto a nylon membrane and UV cross-linked. The membrane was pre-hybridized with 20ml DIG Easy Hyb for 30min at 50°C and hybridized with the DIG-labelled p73 cDNA probe at 50°C for 4hr. After 2 washings (2 X SSC/0.1% SDS, room temperature, 0.5 X SSC/ 0.1% SDS, 68°C), hybridized probe was visualized by an anti-DIG antibody conjugated to alkaline phosphatase and CSPD, chemiluminescent substrate.

Cloning of the ΔNp73 expression plasmids.

PCR was carried out with 100ng of cDNA from JVM-2 in a 50 μl reaction with Expand High Fidelity PCR System enzyme mix (Roche, Basel, Switzerland) according to manufacturer's protocol. Amplification consisted of 1 cycle at 94°C for 2 min, followed by 35 cycles of 94°C for 15 sec, 59 °C for 30 sec, and 72°C for 1,5 min with a cycle elongation of 5 sec for cycles 11-35, and a final elongation at 72°C for 7 min. The primers were as follows: 5'-ATGCTGTACGTCGGTGACCC-3' and 5'-TCAGTGGATCTCGGCCTCC-3' which allowed the amplification of different C-terminal splice variants. The PCR product was cloned into the pcDNA3.1/V5-His Vector (TA Cloning Kit®, Invitrogen, Groningen, The Netherlands) according to the manufacturer's protocol and ΔNp73α, ΔNp73β, and the ΔNp73γ variants were sequenced completely in both directions. Point mutations on the DBD (TAp73-R292H and ΔNp73-R244H) were done using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with mutagenic primers (GTCCTTTGAGGGCCACATCTGCGCCTGTC sense and antisense) according to manufacturer's protocol. The same method was used for mutations of the start codons. For ΔNp73-ATG1m primers were GAATTGCCCTTCTGCTGTACGTCGGTGA sense and antisense, for ΔNp73-ATG2m primers were CTGAGCAGCACCCTGGACCAGAT sense and antisense. Subcloning in pEGFP vector (Clontech) was performed by PCR. PCR products of the open reading frames of TAp73 and ΔNp73 were ligated by T/A cloning using SmaI restriction enzyme (Roche) and the Rapid DNA Ligation Kit (Roche).

Cloning of the ΔNp73 promoter region

Amplification of the 5' upstream region of ΔNp73 was performed using primers: with F1, 5'-GCTGGGCCTTGGGAACGTT-3' and R1, 5'-GGCAGCGTGGACCGAGCGG-3' (construct A) designed on the genomic sequence of clone AL136528, with High Fidelity Taq (Life Technologies). Amplification consisted of 1 cycle at 94°C for 3 min, followed by 40 cycles of 94°C for 45 sec, 60 °C for 45 sec, and 72°C for 2 min, and final

elongation at 72°C for 7 min on a GeneAmp PCR 9600 (Perkin Elmer, Rothrist, Switzerland).The fragment was first cloned into PCR 2.1 Invitrogen, then digested Xho-1 Hind-III and cloned into pGL3basic vector (Promega).

Deletion constructs were generated by PCR. Different forward primers (Construct B: GTTGGAAGGAAAGGGGAAAG; Construct C: ACAC CATCTCTCCCCCTTG; Construct D: CCCTGGTGGGTTTAATTATGG; Construct E: CCCGGACTTGGATGAATACT; Construct F: AAGCGA AAATGCCAACAAAC) and a common reverse primer (GACGA GGCATGGATCTGG) were used. Amplification was carried out with Taq DNA polymerase (Roche) using an annealing temperature of 55°C and 35 cycles. Ligation into the pGL3basic vector was performed by T/A cloning using SmaI restriction enzyme (Roche) and the Rapid DNA Ligation Kit (Roche).

Deletion of the p53 binding site on the A and B construct (A.del and B.del respectively) were performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) with mutagenic primers (CAACACATCACCCGGACTTGGATGAAT sense and antisense) according to manufacturer's protocol.

Dual luciferase-assay

10^5 Saos-2 cells were plated and transfected with Lipofectamine 2000 (Life Technologies, Basel, Switzerland) according to the manufacturer's protocol. Indicated amounts of a reporter plasmid containing firefly luciferase under control of the $p21^{WAF1/Cip1}$ promoter or ΔN promoter described below, together with p53, TAp73 and ΔNp73 expression plasmids were used for transfection. A total of 2μg of plasmids were transfected using pcDNA3 (Invitrogen) to adjust for equal amounts. In all cases 10ng of a renilla luciferase expression plasmid (pRL-CMV, Promega) was co-transfected to normalize for transfection efficiency. All transfections were done in triplicates and the Dual-Luciferase reporter assay system (Promega)

was carried out after 24h from transfection according to the manufacturer's protocol.

Western blot analysis.

Proteins were extracted after tissue or cells disruption, homogenization and sonication for 5min in RIPA buffer (150mM NaCl, 1.0% NP-40, 0.5% DOC, 0.1% SDS, 50mM Tris, pH 8.0); they were quantified by the Bradford method (Bio-Rad, Glattbrugg, Switzerland). Equal amounts of protein (30μg/lane) were size-fractionated on an 8% SDS-polyacrylamide gel, blotted onto nitrocellulose (Protran; Schleicher and Schuell, Dassel, Germany) and checked for equal transfer by staining with Ponceau S. Membranes were blocked with 2% dry milk/TBS and Tween-20 (0.05% v/v) for 1 hr, incubated overnight at 4°C with a 1:500 dilution of a rabbit polyclonal anti-p73 antibody (kindly provided by Dr. D. Caput, Sanofi Recherche, Labège, France). Antibody binding was detected by peroxidase-conjugated goat anti–rabbit antibodies and generation of chemiluminescence by ECL according to the manufacturer's specifications (Amersham, Zurich, Switzerland). X–ray films were used to create a permanent record. Thereafter, the same membranes were stripped, blocked and incubated overnight at 4°C with a 1:1000 dilution of a rabbit polyclonal β-actin antibody (A-2066, Sigma); they were visualised as above. As standards for the Western blots, ΔNp73α/β, and TAp73α/β (the latter 2 subcloned from pcDNA3-HA plasmids (De Laurenzi et al. 2000 neuronal differentiation)) were synthesized *in vitro* by TNT T7 Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's protocol. To assess for protein quality, 30μg of protein was fractionated and blotted as above, and detected with a rabbit polyclonal antibody against actin (A2066; Sigma).

DNA damage induces the rapid and selective degradation of the ΔNp73 isoform, allowing apoptosis to occur (Fig. 15 to 24).

Cell culture and treatments.

H1299, HaCaT and SK-N-SH cells were grown in a mix of MEM (Minimal Essential Medium) and Ham's F12 (1:1, w:w), supplemented with 15mM Hepes, 1,2g/L Sodium Bicarbonate,, and 10% (v:v) of fetal bovine serum. Inducible cell lines (SAOS-2-p53, TAp73α, TAp73β, TAp73γ, ΔNp73α, TAp63α and ΔNp63α) were grown in the same medium mix, supplemented with a tetracycline-free fetal bovine serum (BD Tet-On™ System, Clonetech). SK-N-SH differentiation was induced by a 5 day treatment of all-trans Retinoïc Acid 5μM. "Proliferating" HaCaT were grown without any treatment, "differentiating" HaCaT cells were treated with Ca^{++} 1,2mM for 5 days.

Inducible cell line generation

To generate the SAOS-2(HA-ΔNp73α) inducible cell line, we used the BD Tet-On system (Clonetech), based on the Tetracycline (Tet) Repressor protein regulation. In E.coli, the Tet repressor protein (TetR) negatively regulates the genes of the tetracycline-resistance operon on the Tn10 transposon. TetR blocks transcription of these genes by binding to the tet operator sequences (tetO) in the absence of Tetracycline. TetR and tetO provide the basis of regulation and induction for use in mammalian experimental systems.

The first critical component of the Tet Systems is the regulatory protein**,** based on TetR. In the BD Tet-Off System, this 37-kDa protein is a fusion of amino acids 1–207 of TetR and the C-terminal 127 a.a. of the Herpes simplex virus VP16 activation domain (Triezenberg et al., 1988). Addition of the VP16 domain converts the TetR from a transcriptional

repressor to a transcriptional activator, and the resulting hybrid protein is known as the tetracycline-controlled transactivator (tTA). tTA is encoded by the pTet-Off regulator plasmid, which also includes a neomycin-resistance gene to permit selection of stably transfected cells. The BD Tet-On system (the one used in our inducible cell line) is similar to the BD Tet-Off system, but the regulatory protein is based on a "reverse" Tet repressor (rTetR) which was created by four amino acid changes in TetR (Hillen & Berens, 1994; Gossen et al., 1995). The resulting protein, rtTA (reverse tTA), is encoded by the pTet-On regulator plasmid, which also contains a neomycin-resistance gene.

The second critical component is the response plasmid (pTRE or its variants), which expresses a gene of interest (in this case, HA-ΔNp73α) under control of the tetracycline-response element, or TRE. The TRE consists of seven direct repeats of a 42-bp sequence containing the tetO, and is located just upstream of the minimal CMV promoter (PminCMV), which lacks the strong enhancer elements normally associated with the CMV immediate early promoter. Because these enhancer elements are missing, there should not be "leaky" expression of HA-ΔNp73α from the TRE in the absence of binding by the TetR domain of tTA or the rTetR domain of rtTA. The ultimate goal in setting up a functional Tet System is creating a double stable Tet cell line which contains both the regulatory and response plasmids. When cells contain both the regulatory (pTet-On) and the response (pTRE-HA-ΔNp73α) Vectors, HA-ΔNp73α is only expressed upon binding of the rtTA protein to the TRE. In the BD Tet-On System, rtTA binds the TRE and activates transcription in the presence of Doxycycline. In both BD Tet-On and Tet-Off Systems, transcription is turned on or off in response to Doxycycline in a precise and dose-dependent manner.

The SAOS-2(pTet-On), kindly provided by Karen Vousden, were transfected with the pTRE-HA-ΔNp73α vector, containing the Hygromycine resistance gene. Two days after transfection, cells were splited at diverse confluence rates and selected with Hygromycine 300μg/mL for at least 2 weeks, when the first resistant clones appear. Each clone is first grown in a 96 wells plate and then amplified in T75 cm^2 flasks, at least 30 clones are choosen. Lots of clones die or are eliminated because of their too low or too

active proliferation rate. 8 clones were analysed by immunofluorescence staining with the confocal microscope, 2 of them presented a cytoplasmic localization of the protein, and 2 others were “leaky” (the protein was expressed even without induction).

Analysis of apoptosis.

To estimate DNA fragmentation, H1299, HA-TAp73α or HA-ΔNp73α SAOS-2 cells were plated to approximately 50% confluency and irradiated with UV (140J/m^2) and/or induced with Doxycyclin for 6h, 24h, 48h or 72h. Cells were collected at 800g for 10 min and fixed in 70% ethanol. Hypodiploid events were evaluated by flow cytometry using a propidium iodide (PI) staining (40 mg/ml) in the presence of 13 kU/ml ribonucleaseA (20 min incubation at 37°C) on a FACS-Calibur flow cytometer (Becton-Dickinson, CA, USA). Cells were excited at 488 nm using a 15mW Argon laser, and the fluorescence was monitored at 578nm at a rate of 150 ± 300 events / s. Ten thousand events were evaluated using the Cell Quest Program (ibid). Electronic gating FSC-a/vs/FSC-h was used, when appropriate, to eliminate cell aggregates.

Immunofluorescence analysis

Induced SAOS-2(HA-ΔNp73α) or (HA-TAp73α), or HaCaT cells, grown on cover glasses in 12 wells dishes, were fixed with Paraformaldehyde 4% and Picric Acid 0,19% in PBS 1X for 45min. After washes in PBS, cells were permeabilized 10min with SDS 0,1% in PBS and then blocked in Fetal Calf Serum 10% for 20min. After a wash in PBS, cells were incubated 1hour either with anti-HA antibody (1:500 Santa Cruz Y-11), or with our polyclonal anti-p73 antibody (1:1500) in PBS-BSA 1mg/mL.They were washed again, and incubated with a goat anti-mouse antibody conjugated with a red or green fluorescent peptide in PBS 1mg/mL for 30min. Slide were analysed with a confocal microscope.

Polyclonal anti-p73 antibody characterization

Adult rabbit was immunized by injection of a p73 core domain peptide, conjugated with BSA (bovine serum albumin). Serum was taken and used for the antibody characterization, without further purification.

For over-expression analysis, induced SAOS-2(p53, HA-TAp73α,β,γ, HA-ΔNp73α, HA-TAp63α and HA-ΔNp63α). Cells were trypsinised, pelleted and washed with PBS. The pellet was resuspended in lysis buffer (50mM Tris-HCl pH 7.5, 500mM NaCl, 1% TX-100, 0.5% NP-40, 0.5% sodium deoxycholate, 1mM DTT, 1mM EDTA and 1mM AEBSF) and sonicated. The homogenate was kept for 1 hour in ice, and then centrifuged at 10.000xg for 30 minute at +4°C. The supernatant was recovered and used for western blot analysis. 10μg of protein were loaded using the new p73 antibody, while 50μg of protein were loaded using Ab2 and Ab4 commercial antibodies. Proteins were separated on a 10% SDS-polyacrylamide gels and blotted onto PVDF membrane. Filters were blocked with 10% non-fat dry milk and 5% BSA for 2hours, and then incubated for 2hours with new p73 antibody (diluted 1:5.000 in blocking solution) or Ab2 and Ab4 antibodies (1:100 in blocking solution). After 3 washes with PBS+0.1% tween-20, filters were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:20.000 in blocking solution) for 1hour. Detection was performed using West Dura ECL system (Pierce).

For endogenous p73 protein analysis, nuclei from SK-N-SH and HaCat cells were purified as described below. 100μg of nuclear proteins were loaded onto a 10% SDS-polyacrylamide gel and blotted onto PVDF sheets. Filters were blocked with 10% non-fat dry milk and 5% BSA for 2 hours, and then incubated for 2 hours with new the p73 antibody (1:3.000 in blocking solution). Detection was performed as above.

For p73 immunoprecipitation, SAOS-2(HA-TAp73α) cells were induced with doxycycline for 24 hours and nuclei were purified as described below. 200μg of nuclear proteins were diluted in “binding buffer” (25mM Tris-HCl pH 7.5, 250mM NaCl, 0.5% TX-100, 0.25% NP-40, 0.25% sodium deoxycholate, 1mM DTT, 1mM EDTA and 1mM AEBSF) and incubated

over-night with 25µl of equilibrated protein A agarose-conjugated beads (Amersham), 1or 2µl of new p73 antibody or 10µl (2µg) of Ab4 antibody. Beads were then pelleted (10000g for 1 minute) and washed 3 times with binding buffer. Immunoprecipitated proteins were eluated by resuspending agarose beads in 50µl of Laemli Buffer and boiling at 100°C for 10 minutes. 10µl of the supernatant were loaded in 10% SDS-polyacrylamide gels and blotted onto PVDF sheets. Immuno-detection was performed using anti-HA antibody (Santa Cruz Y-11) diluted 1:500 in PBS-tween for 1 hour.

Western-Blotting

Protein extraction was performed in 10mM Tris-HCl pH7,5, 150mM NaCl, 1%SDS, 1%NP40, EDTA 1mM, DTT 2mM, Protease Inhibitor Cocktail (SIGMA). 30µg of total cellular protein was size-fractionated in Laemli Buffer on an 10% SDS polyacrylamide gel and blotted onto a PVDF membrane. Equal loading and transfer efficiency were assessed by ponceau staining. Anti-HA (BABCO HA-11**,** dilution 2µg/mL, or Santa Cruz, clone Y-11), or the new anti-p73 antibodies were used for the detection.

Nuclear extraction

H1299, HaCaT, SK-N-SH cells were harvest in Cell Lysis Buffer (10mM HEPES, 10mM NaCl, 1mM KH_2PO_4, 5mM $NaHCO_3$, 1mM $CaCl_2$, 0,5mMMgCl_2, 5mM EDTA), and dounce homogenized 50 times. After centrifugation, pellet was resuspended in TSE buffer (10mM Tris pH7,5, 300mM sucrose, 1mM EDTA), and dounce homogenized 30 times. After two washes, nuclei were resuspended in TSE 1%NP40, sonicated and 30µg nuclear protein was analysed by immunoblotting.

Quantitative RT-PCR

The HA-TAp73α and HA-ΔNp73α inducible cell lines were treated with Doxycycline for 18h. Doxycycline was removed, cells were irradiated (140J/m2) and harvested after 1, 3 or 6h. mRNA were extracted with RNeasy MiniKit© (QUIAGEN) according to the manufacter's protocol. RT-PCR was performed with Random Primers and HA-TAp73 and HA-ΔNp73 levels were assayed with PCR using HA-Forward 5'ATACGATGTT CCAGATTAGG3' and Reverse 5'CGGGGTAGTCGGTGTTGGAG3' primers. β-Actin served as an internal control for the reverse transcription and was amplified from the same cDNA as described above : Forward primer 5'CTGGCACCACACCTTCTACAATG 3', Reverse primer 5'AATG TCACGCACGATTTCCCGC3'. PCR products were separated on a 2% agarose gel and stained with ethidium bromide.

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CURRICULUM VITAE & PUBLICATIONS

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Present position

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Education

2000 : Degree in Biology (5 year course), Master in Genetics (3 year course). Final score 16/20.

1994 : General certificate of education, equivalent to A level (final score 15,96/20).

Previous positions and scientific interests

10-2000 until present : Transcriptional and post-translational regulations of the ΔNp73 protein.

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Technical experience

Cell culture and transfection, creation of inducible cell-lines, flow cytometry. EMSA, cell-free systems (nuclei and mitochondria), protein extraction, immunoprecipitation and Western blot. PCR amplification, DNA cloning, recombinant and fusion protein expression. Promoter study by luc-assay and mutagenesis.

Language ability

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PUBLICATIONS

1. **Maisse C,** Munarriz E, Barcaroli D, Melino G and De Laurenzi V
   DNA damage induces the rapid and selective degradation of the ΔNp73 isoform, allowing apoptosis to occur.
   Cell Death Differ. In press.
2. **Maisse C**, Bernassola F, Salomoni P and MacFarlane M.
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