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Review

p73: A *chiaroscuro* gene in cancer

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ABSTRACT

p73 is a member of the p53 family which is gaining increasing importance in the field of cancer. Its structural homology with p53 led to the assumption that it could act as a new tumour suppressor gene. Increasing knowledge of its function, however, has cast doubts on this role. A particularly interesting characteristic of p73 is that the cell contains different isoforms with distinct and sometimes opposite functions. Evidence in the last few years clearly indicates that p73 does share some activities with p53 but also that it has some distinct functions.

This review focuses on p73's role in the development and progression of cancer, analysing the gene structure and regulation and discussing similarities with p53 and differences. Recent results obtained with specific detection methods on the levels and functions of the different isoforms in tumours are also discussed.

1. Introduction

One of the most important tumour suppressor genes in human cancer is p53. In human tumours it is often mutated and the wild type p53 helps maintain normal cell division and cell death, preventing the development of cancer. Discovered in 1979,[1,2] p53 was long assumed to be encoded by a single gene translated in a single protein with no structural and functional homologues. Only recently, the group headed by David Lane reported that the *p53* gene contains an internal promoter and can encode at least six different isoforms, expressed in normal cells and breast tumours in a tissue-dependent manner.[3]

Since the discovery several years ago of two *p53*-related genes, *p63* and *p73*, we now know that *p53* is not alone but belongs to a family. This review will be mainly centred on *p73*, which was discovered in 1997.[4] Data on p73 are sometimes controversial but we shall try to establish whether it really plays a role in tumour prevention and in the cellular response to anticancer treatments, as p53 does.

2. Structure of p73 and splicing variants

The structural organisation of p73 is similar to p53. All the p53 family members share three major functional domains: the NH_2-terminal transactivation domain, the central

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sequence-specific DNA binding domain, and the COOH-terminal oligomerisation domain (Fig. 1a). The central DNA binding domain is highly conserved across the family.[5]

p73 is expressed in multiple variants arising from alternative splicing of the primary p73 transcript including the C-terminal isoforms p73α, p73β, p73γ, p73δ, p73ε and p73ζ.[4,6,7] p73α is the longest form, containing a sterile α motif domain (SAM) in the extreme COOH-terminal region. All other isoforms are rearranged in the COOH-tail and lack the SAM domain (Fig. 1b). These splicing variants are expressed differently in normal human tissues and cell lines. Besides TAp73, four different NH_2 terminally truncated isoforms, ΔNp73, Δ′Np73, ΔEx2p73 and ΔEx2/3p73, have been found in human cancers and cancer cell lines. Each one lacks all or most of the transactivating domain so they are collectively called DNp73 (Fig. 1b). *DNp73* products are generated either through alternative exon splicing of the P1 promoter transcript (producing Δ′Np73, ΔEx2p73, and ΔEx2/3p73), or by use of the P2 promoter in intron 3, producing ΔNp73. The ΔEx2p73 and ΔEx2/3p73 isoforms lack exon 2 and exon 2/3, respectively. The transcripts ΔNp73 and Δ′Np73 encode the same protein product.

3. Specific activity of p73 splicing variants

p73 C-terminal splicing variants differ in their ability to induce p53 target genes. According to the literature, p73β is a more potent inducer than the other variants.[7,8] In spite of the similarities, p73 definitely has some different activities and that help explain the differences in behaviour. There are reports of activity exerted by p73 but not by p53: the cyclin-dependent kinase inhibitor p57 KIP2, which is a transcriptional target of p73β but not of p53, is one example.[9] p73β enhances Wnt/beta-catenin signalling by activating the promoter containing the Tcf-binding element; p73β and p63 but not p53 can induce the WNT4 promoter.[10] In addition, interleukin 4 alpha, which may mediate certain immune responses, is regulated by p73 but not significantly by p53 in several human cancer cell lines.[11] Probably many other genes can be specifically induced only by p73 and not by p53.

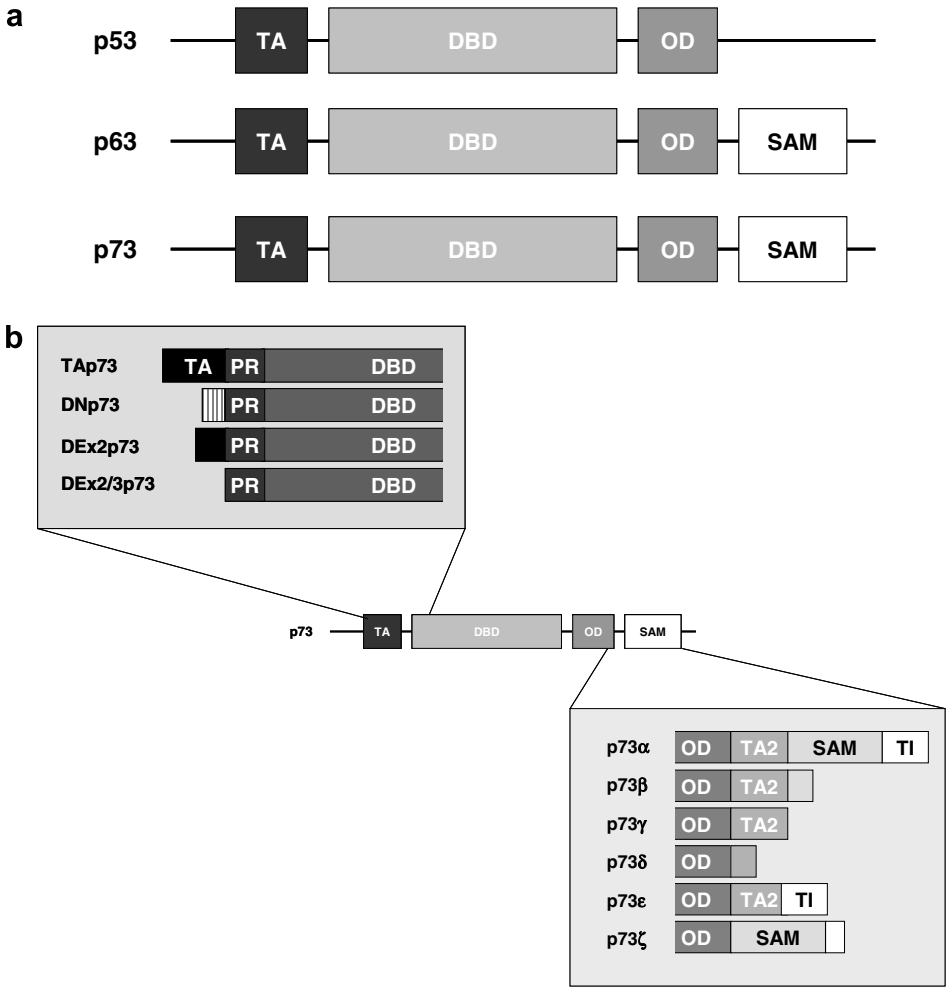

Fig. 1 – Panel a: Schematic structure of the p53 family. All members have transactivation (TA), DNA binding (DBD) and oligomerisation (OD) domains. The sterile alpha motif (SAM) domain is peculiar to p63 and p73. Panel b: Schematic drawing of the NH2 and COOH terminal splicing ends of p73.

Most reports suggest that DNp73 isoforms do not cause cell cycle arrest and apoptosis. Their biological importance might lie in the fact that DNp73 proteins retain their DNA-binding, tetramerisation competence and can thus act as dominant-negative inhibitors of both wild-type p53 and TAp73. However, all the different DNp73 isoforms can theoretically be generated with a different C-terminal end, increasing the number of potentially relevant isoforms.

This viewpoint was challenged by the recent finding of Liu and colleagues[12] that ΔNp73β has weak but distinct transcriptional activity. This isoform raised the endogenous MDM2 and p21 level in the SaoS2 cell line and induced the GADD45 promoter, leading to cell cycle arrest or apoptosis. In contrast, ΔNp73α could not cause cell cycle arrest or apoptosis under the same experimental conditions. Liu's results suggest that the NH_2-terminal 13 unique amino acid residues, the PXXP motifs and the lack of SAM domain in ΔNp73β might be a novel activator factor (Fig. 2 panel e). Thus, ΔNp73β might have distinct effects on certain cellular processes.

ΔNp73α also has transcriptional activity. Heat shock factor (HSF)-responsive gene expression was selectively activated by ΔNp73α, but not by other isoforms.[13] Apart from P1 and P2 promoters, the 5′ region of TAp73 may also comprise a region within the first intron of the p73 gene,[14] which could be a putative new promoter region. However, there is as yet no information on the function of this promoter.

4. Role in development and cancer as suggested by knock-out mice

Gene targeting studies in the mouse indicate that p73 has a role in normal development.[15,16] The construct utilised to generate KO mice deleted the central DNA binding domain of p73 so they affect all the different isoforms of the gene. p73 mutant mice survive birth but $p73^{-/-}$ pups have a runting phenotype and high rates of mortality. Most commonly, death follows massive gastrointestinal haemorrhages, although intracranial bleeding is also apparent in 15%. Histological examination of the gastrointestinal tract of these mice on postnatal day 7 (P7) shows numerous abnormalities including erosion, with a loss of enterocytes and excessive mucosecretion in the duodenum, ileum and cecum, which may underlie the wasting syndrome and the intestinal bleeding. The majority of p73-deficient mice only live to be 4 to 6 weeks old and die of chronic infections.[16] A striking feature is severe rhinitis and purulent otitis media. They also have mild congenital hydrocephalus that progresses in some animals to a highly morbid condition with massive expansion of the lateral ventricles, compression of the cortex and intraventricular bleeding. These mice have a communicating form of hydrocephalus, meaning there might be defects in the production or reabsorption of cerebrospinal fluid.[16] The hippocampal dysgenesis and defects in sensory or hormonal pathways may contribute to the reproductive and behavioural phenotypes of p73-deficient mice. The apparent requirement for p73 in such processes as pheromone detection, neurogenesis and fluid dynamics illustrates its role in sensing environmental and homeostatic stimuli.[16]

In contrast to p53-deficient mice, however, those lacking p73 show no increase in susceptibility to spontaneous tumourigenesis. The absence of tumours in *p73* ko mice seriously pointed to a lack of tumour suppression function. Their lifespan of around 4–6 weeks is probably not enough for tumours to develop but Flores and colleagues[17] investigated the long-term tumourigenic effects of *p73* heterozygous mutation in mice, alone or with p53. After 2 years, 36 out of 40 $p73^{-/+}$ mice had been euthanised or had died, compared to 8 of the 40 in the wild-type group. A full autopsy was done on all animals to check the frequency and spectrum of spontaneous tumours; $p73^{+/-}$:$p53^{+/-}$ background mice developed a more aggressive tumour phenotype than the $p73^{+/+}$:$p53^{+/-}$ animals. These data suggest that p73 is important in tumour suppression in specific tissues in the mouse.

5. Level of p73 and frequency of mutations in normal tissues and cancer cells

Initially it was reported that p73 is expressed at a very low level in normal human tissues[18] and the same was shown in normal human thymocytes.[19] However, hyperexpression of p73 in many different human cancers was also described.[20] p73 was over-expressed in tumours such as ependymoma, breast, neuroblastoma, lung, oesophagus, stomach, colon, bladder, ovary, hepatocellular carcinoma, myeloid leukaemia. When these studies were done, DN isoforms of p73 were not known and the hyperexpression in cancer represented the overall expression of p73 mRNA. Mainly quantitative reverse transcription-polymerase chain reaction methods were used.[21] In addition, antibodies to distinguish the different isoforms were not available then.

In 2000 DNp73 was discovered in mice neurons[22] and later it was shown that it is also expressed in cancer cells.[23] With this evidence, specific primers to differentiate full-length TAp73 from DNp73 were used to measure p73 levels in tumours. It became clear that very often TAp73 and DNp73 can co-express in tumours[24] so dominant negative DNp73 isoforms rather than TAp73 might be physiologically more important components of p73 over-expression in tumours.[25]

The DNp73 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. This inhibitory function is exerted either at the oligomerisation level, or by competing for binding to the same DNA target sequence (Fig. 2).[26–28] DNp73 isoforms can therefore control the activity of both TAp73 and p53. In addition, the *DNp73* promoter contains a very efficient p53/TAp73 responsive element of particular interest.[29,30] p53 and TAp73 can induce expression of the DNp73 isoform, and therefore create a dominant-negative feedback loop that regulates the function of both p53 and TAp73; it can fine-tune the function of p53 in a manner similar to the MDM2 loop.[31] Both *MDM2* and *DNp73* are direct transcriptional targets of p53, and inhibit p53 function by inducing its degradation (in the case of MDM2) or competing for its target DNA-binding sites (DNp73). Perturbations of these regulatory loops in cancer cells[32] or in virally infected cells,[33] causing excess or persistent expression of MDM2 or the ΔNp73 isoform, might result in an inability to activate p53 or TAp73. Consequently, loss of these

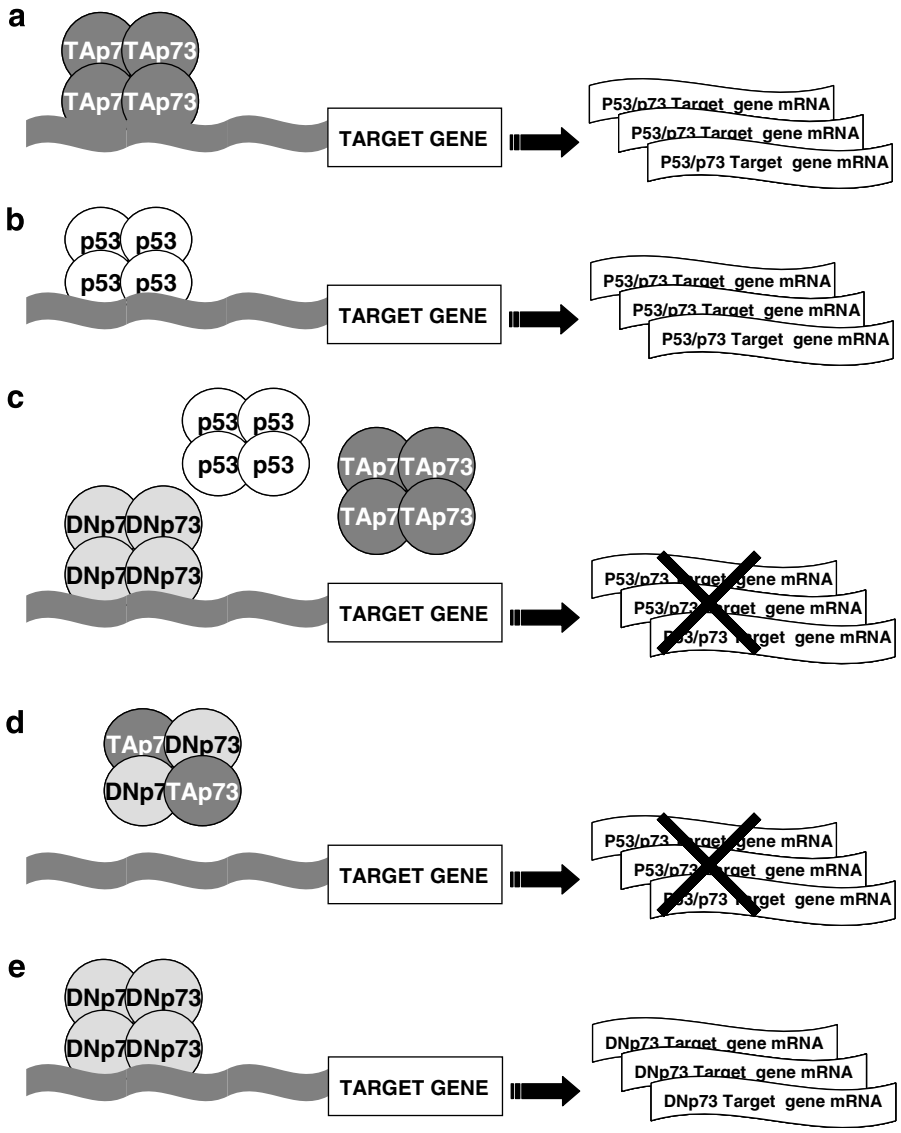

Fig. 2 – DNp73's role in p53- and p73-proficient cells. In the absence of DNp73, Tap73 and p53 can activate the transcription of p53/TAp73 target genes (panels a and b). DNp73 is able to block the activity of TAp73 and p53 by occupying the DNA binding site (c) or by forming an inactive hetero-tetramer with transcriptionally proficient proteins (d). Data is emerging that DNp73 can act as a true transcription factor (e).

regulatory pathways might allow inappropriate p53 or TAp73 activity and inhibition of cell growth, contributing to cancer development.[34,35] Indeed, deletion of *MDM2* results in early embryonic lethality that directly depends on high p53 activity.[36,37]

The potential oncogenic function of ΔNp73 is also indicated by the observation that over-expression of the ΔNp73 isoform results in malignant transformation of NIH-3T3 fibroblasts and tumour growth in nude mice.[38] Probably in the presence of DNp73, TAp73 might be inactivated, as described for human thyroid cancer, so even if it is over-expressed it fails to induce either cell-cycle arrest or apoptosis.[19] The reason for over-expression of TAp73 or DNp73 is not clear. It might be related to an increase in transcriptional activity of P1 and P2 promoters, to the stability of mRNA, or to the steady state of proteins by changes in post-translational modifications.

Since p53 is very often mutated in human tumours many investigations have looked for mutations in the p73 gene. At present it appears that this gene is very rarely mutated and in fact mutations were detected in fewer than 0.5% of human cancers, whereas over 50% carry p53 mutations. In conclusion, p73 is very often over-expressed in cancer, implying that it may be present but not transcriptionally active. Mutations in the *p73* gene were very infrequent in various tumours. All these findings argue against a role of p73 as a classic tumour suppressor.

6. Transcriptional regulation of TAp73 and DNp73

All the upstream signals of p73 that operate physiologically need to be identified to understand its role in normal cell life

and, possibly, in tumourigenesis. With a view to explaining the reasons for p73 hyperexpression in human tumours, the P1 promoter of p73 was cloned[39] but no similarities were found between human p73 and p53 promoters. Several E2F potential binding sites were identified which in the p73 promoter might have a role in p73 transcriptional regulation. The members of the E2F family of transcription factors are key regulators of genes involved in cell cycle progression, cell fate, DNA damage repair, and apoptosis. Many cell-based experiments suggest that E2F1 is a stronger inducer of apoptosis than all the other E2Fs.[40]

In 2000, three papers were published independently providing evidence that E2F1 directly activates transcription of p73 which, in turn, leads to the activation of p53-responsive target genes and apoptosis.[41] Thus, p73 activation by deregulated E2F1 activity might form a p53-independent, anti-tumourigenic safeguard mechanism.[42] The region conferring the highest promoter activity resides between positions −113 and −217 (relative to the transcription starting site) of the *p73* gene. Two of the three functional E2F sites reside within this region (at −155 and −132). Thus regulation of p73 expression may primarily involve E2F1 binding to target sites at −155 and −132.[43]

Besides E2F1, several other transcriptional factors may be involved in p73 regulation. Over-expression of cellular and viral oncogenes up-regulates endogenous p73 proteins and activates their transcriptional functions.[21] There are also several investigations of down-regulation of p73. p73 mRNA is undetectable in proliferating C2C12 cells and is expressed at very low levels in undifferentiated P19 and HL60 cells.[44] MEK1 inhibitors can promote the accumulation of endogenous p73 through its transcriptional activation.[45]

The P1 promoter can be regulated by p53 through the p53 potential binding site.[46] However, this putative p53 element was not responsible for the DNA damage-dependent activation of p73.[47] It is important to stress that activation of the P1 promoter is responsible not only for the transcriptional up-regulation of TAp73 but also for the up-regulation of the aberrantly spliced ΔN′p73 isoforms (at least those transcribed through this promoter).

The transcriptional factor C-EBPα seems to have direct repressive activity on transfactor E2F1. In normal conditions a repressor complex involving C-EBPα, E2F1 and perhaps other proteins is present on the p73 promoter. This complex is destroyed after damage by removal of C-EBPα from nuclei and p73 mRNA is induced in an E2F1-dependent manner after damage by this mechanism.[47]

A 1-kb regulatory fragment was identified within the first intron of p73, which is immediately upstream of the ATG codon of the second exon. This fragment exerts p73 silencer activity on heterologous promoters. The p73 intronic fragment contains six consensus binding sites for transcriptional repressor ZEB, which binds these sites both *in vitro* and *in vivo*.[44]

Human DNp73 lacks the transactivation domain and starts with an alternative exon (exon 3′). Its expression is driven by a second promoter, P2, located in a genomic region upstream of this alternative exon, supporting the idea that TAp73 and ΔNp73 are two independently regulated proteins, derived from the same gene. As was expected, DNp73 is capable of regulating TAp73 and p53 function since it can block their transactivation activity and their ability to induce apoptosis. After cloning the P2 p73 promoter it became clear that expression of ΔNp73 was strongly up-regulated by the TA isoforms and by p53, thus creating a feedback loop that tightly regulates the function of TAp73 and, more importantly, of p53. The expression of DNp73 not only regulates the function of p53 and TAp73 but also shuts off its own expression. ΔNp73 is regulated through a p53-responsive element in the p73 P2 promoter.[48]

7. Steady-state regulation of TAp73 and DNp73

Because of the structural homology between p53 and p73, and considering that p53 levels are regulated by MDM2, this E3 ubiquitin ligase was considered as a natural controller of p73 degradation. MDM2, in fact, controls p53 activation through a finely tuned mechanism in which p53 both induces MDM2 and is degraded by it.[49] This mechanism, however, does not apply for p73 because it lacks homology to the 92-112 region of p53, which is the MDM2 interaction site. Intriguingly, MDM2 does bind p73, but this interaction increases the stability of p73. A newly identified p53-induced E3 ubiquitin protein ligase, Pirh2, which stimulates the ubiquitination-dependent proteolytic degradation of p53, had negligible effect on p73.[50]

The transcriptional activity of p73 might play a role in protein stability and its degradation could depend on the induction of some unknown E3 ubiquitin protein ligase. p73 deletion or point mutants that lack transactivation activity were more stable than wild type p73. The naturally occurring p73 variant ΔNp73 was also more stable than TAp73.[51] Indeed, ΔNp73, which potentially inhibits the transcriptional activity of TAp73, can boost TAp73 protein stability.[52] Stability of p73 is not only transcriptionally dependent but also transcriptionally independent.[53] The binding of p73 to Itch, a Hect ubiquitin-protein ligase, has been described. Itch selectively binds and ubiquitinates p73 but not p53; this results in the rapid proteosome-dependent degradation of p73.[54]

Finally, the regulation of p73 degradation requires the integrity of the NQO1 pathway. This is an ubiquitin-independent process mediated by the 20S proteasome that is regulated by the 20S gatekeeper NQO1 protein[55] (Fig. 3). The steady state of p73 can also be modified by Sumo-1, an ubiquitin-like protein, first identified as a GAP-modifying protein. The sumoylation of p73α might influence interactions with other proteins, such as c-Abl tyrosine kinase,[56] that can stabilise p73. The presence of p73 in PML bodies (also called nuclear bodies) has raised the possibility that p73 interacts with PML in these bodies, thus regulating its stability.[57]

During the search for molecules interacting with the COOH-terminal proline-rich region of p73, a novel NEDD4-related protein was identified, known as NEDL2. NEDL2 catalyses the ubiquitination of bacterial cellular proteins *in vitro*. Reciprocal co-immunoprecipitation experiments and *in vitro* pull-down assays showed that NEDL2 can bind to p73. p73 was efficiently ubiquitinated, but stabilised, in a NEDL2-dependent manner. Thus p73 decayed faster in the absence

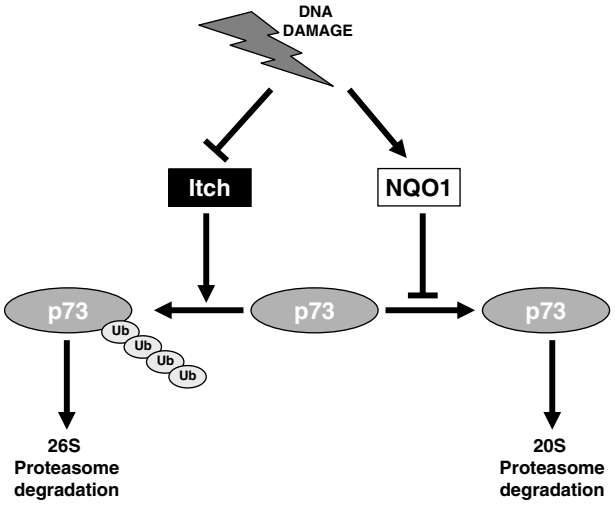

Fig. 3 – Schematic representation of the ubiquitin-dependent and ubiquitin-independent pathways in p73 degradation.

of NEDL2 than in its presence. Consistent with the stabilisation of p73, NEDL2 enhances p73-dependent transcriptional activation. These results suggest that NEDL2 activates the function of p73 by increasing its stability.[58]

p73 is physically as well as functionally associated with the U-box-type E3/E4 ubiquitin ligase UFD2a. Immunoprecipitation experiments demonstrated that this interaction is mediated by the COOH-terminal region of p73α which contains the SAM domain. During cisplatin-induced apoptosis in SH-SY5Y neuroblastoma cells, p73α accumulates at protein level, while the endogenous UFD2a is significantly reduced. Ectopic expression of UFD2a shortens the half-life of p73α, with significant inhibition of p73α-mediated transactivation and pro-apoptotic activity.[59]

Another protein that might be involved in p73 stability is cyclin G, which is a transcriptional target gene of the tumour suppressor p53. Cyclin G may play a central role in the p53-Mdm2 auto-regulated module, but its precise function remains elusive. It had a negative effect on the stability of p53 and p73. It interacts directly with p53 as well as p73, and its binding to p53 or p73 presumably mediates their down-regulation.[60] c-Jun transcriptional factor can regulate the stability of TAp73 and both the transactivation and the DNA-binding domain appear to be critical for c-Jun-mediated p73 accumulation. No physical interactions were found between c-jun and p73.[61]

Nothing is yet known about possible differences in regulation of TA and DNp73 stability in normal unstressed conditions. It would be exciting to find a way to shorten the half-life of DNp73 which could potentially inhibit TAp73 activity.

Post-transcriptional p73 regulators are represented in Fig. 4.

8. Regulation of p73 transcriptional activity

The transcriptional activity of p73 can be induced by c-Abl, a non-receptor tyrosine kinase activated by DNA damaging agents. C-Abl was the first protein proposed to be involved in regulating p73 activity. p73 and c-Abl can associate with each other, and this binding is mediated by the PxxP motif in p73 and the SH3 domain of c-Abl. In addition, p73 is a substrate of c-Abl kinase, whose ability to phosphorylate p73 is markedly increased by gamma-irradiation.[62,63]

Other activators subsequently started to be connected with p73. Two proteins, ASPP1 and ASPP2, stimulated the apoptotic function of p53. Both ASPP1 and ASPP2 induced apoptosis independently of p53. ASPP1 and ASPP2 bound p63 and p73 *in vitro* and *in vivo*, stimulating the transactivation function of p63 and p73 on the promoters of Bax, PIG3, and PUMA but not those of mdm2 or p21(WAF-1/CIP1).[64]

The MDM2-related protein MDMX also binds p73 and stabilises the levels of p73. In addition, the growth suppression functions of p73 and the induction of endogenous p21, a major mediator of the p53-dependent growth arrest pathway, were enhanced in the presence of MDM2X.[65]

Co-fractionation and co-immunoprecipitation assays investigation of the regulation of p73 transcriptional activity by p300/CREB binding protein (CBP) identified p73-p300 complexes in HeLa cell extracts. The p73-p300 interaction was confirmed *in vitro* by glutathione S-transferase-protein association assays and *in vivo* by co-immunoprecipitating the over-expressed p300 and p73. Over-expression of either p300 or CBP stimulated transcription of p73 while its N-terminally deleted mutant *in vivo* was not.[66]

p73 might also be physically associated with the Yes-associated protein (YAP). This occurs under physiological conditions, as shown by reciprocal co-immunoprecipitation of complexes from lysates of P19 cells. The WW domain of YAP and the PPPPY motif of p73 are directly involved in the association. Unlike p73α, p73β, and p63α, which bind to YAP, the endogenous and exogenously expressed wild-type p53 and the p73γ isoform do not interact with YAP. YAP interacts only with the p53 family members that have a well-conserved PPXY motif, a target sequence for the WW domains. Over-expression of YAP increases p73 transcriptional activity;[67] c-jun, which prolongs the half-life of p73 in co-transfection experiments, also works as a transcriptional activator of p73.[61] p73α is functionally associated with the human homologue of mouse and hamster homeodomain-interacting protein kinase 2 (HIPK2). The hamster homologue, haHIPK2 or PKM, was used to further characterise interactions between HIPK2 and members of the p53 protein family. Systematic yeast two-hybrid assays found a physical interaction between the oligomerisation domains of p73α and p53 (amino acid regions 345–380 and 319–360, respectively) and the amino acid region 812–907 of haHIPK2. HIPK2 co-localises with p73 and p53 in nuclear bodies, as demonstrated by confocal microscopy. Over-expression of HIPK2 stabilises the p53 protein and greatly boosts the p73 and p53 induced transcriptional repression of multidrug-resistant gene and collagenase gene promoters in Saos2 cells but had little effect on the p73- or p53-mediated transcriptional activation of synthetic p53-responsive elements and p21WAF1 promoters.[68] HIPK2 is an example of a protein that increases the transcriptional repressive activity of p73.

WT1 is a gene that represses p73's transactivation abilities. This gene, which is heterozygously mutated or deleted in congenital anomaly syndromes and homozygously mutated in

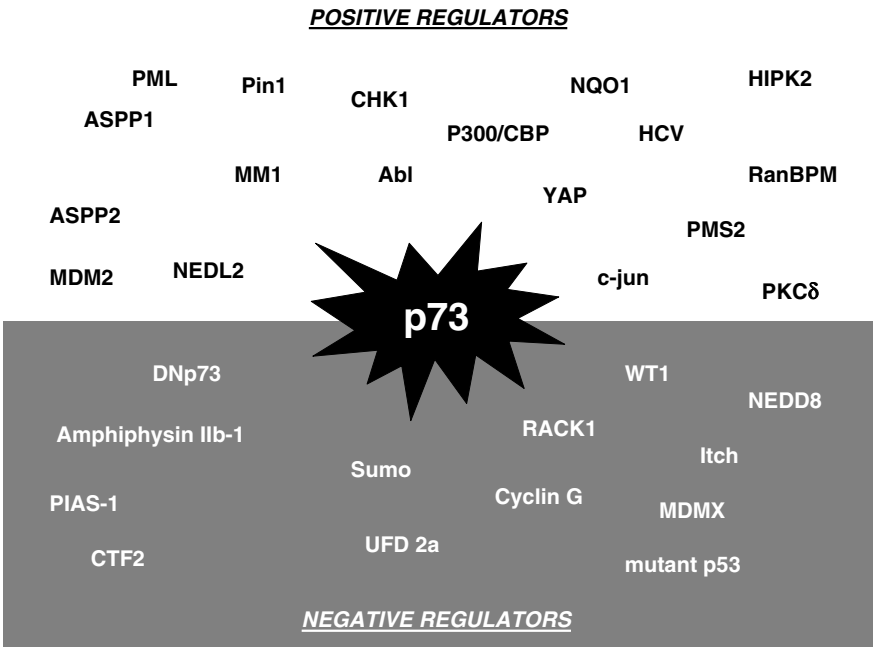

Fig. 4 – Positive and negative activity regulators of p73.

about 15% of all Wilms tumours, encodes tissue-specific developmental regulators. Interaction between WT1 and p53 affected their ability to regulate the transcription of their respective target genes. WT1 binds to p73, and p73 binds to the zinc finger region of WT1, inhibiting DNA binding and transcription activation by WT1. Similarly, WT1 inhibits p73-induced transcriptional activation in reporter assays and counteracts p73-induced expression of endogenous Mdm2.[69] Positive and negative transcriptional activity regulator proteins of p73 are schematically represented in Fig. 4.

9. Interaction between p73 and p53

How can p73 and p 53 co-operate in a cellular context? This question is very important for cells that express both proteins. Initially it was found that p73β, but not p73α, interacted, albeit modestly, with p53 in a yeast two-hybrid assay.[4] Later, when the complex formation between wild-type p53 and p73 was investigated in transfected mammalian cells, no co-precipitation was found between p73 and wild-type p53.[70] The oligomerisation domain of p53 does not associate with either p73 or p63, even when p53 is in large excess.[71] The results were similar in an ovarian cancer cell line over-expressing p73α with endogenous wild-type p53 in our laboratory.[72]

There is contradictory evidence on the functional cooperation between p73 and p53. We previously showed that p73α, a weak activator of p53 downstream genes, can inhibit the transcriptional activity of wild-type p53 by competing for the p53 binding site.[72] The same results were obtained in another work where suppressive effects of p73α, γ and δ, but not β, on endogenous p53 activity were observed when transiently expressed in HepG2 and MCF-7 cells.[7] Other findings indicate that p73 hyperexpression reduces the levels of ectopically expressed p53 in transient transfections or of the endogenous p53 induced by adriamycin- or UV-mediated damage. These reductions were presumably due to an increase in MDM2-mediated proteolysis. These findings suggest that different levels of p73 in the cell may influence p53 responses after DNA damage and other stresses, and that an increase rather than a decrease in p73 may play a role in tumourigenesis.[73]

The relation between p53 and p73 has been analysed using T cell death of primary, genetically defined lymphocytes. The results challenge the notion that p73 is required for p53 function or for apoptosis in the T-cell.[74] At the same time, analysis of genetically defined, E1A-programmed murine embryonic fibroblasts (MEFs) points to functions for p63 and p73 in p53-dependent cell death. In fact, the combined loss of p63 and p73 results in the failure of cells containing functional p53 to undergo apoptosis in response to DNA damage.[75] Other data suggesting functional cooperation between p53 and p73 were obtained in neuroblastoma cell lines. In these tumours with wild-type but non-functional p53, TAp73 activated some p53 responsive genes. From a clinical point of view, p53 'restoration' through TAp73 over-expression may be useful and may offer new therapeutic strategies.[76] The data so far on the interplay between wild-type p53 and p73 are very contradictory, with striking differences, depending on the cellular context.

In approximately 50% of human tumours, there is a mutant form of p53 and several investigations have tried to shed light on its interaction with p73. p73α co-precipitates with mutant p53. Since many tumour-derived p53 mutants inhibit wild-type p53-mediated transactivation, the effects of two representative hot-spot mutants (R175H and R248W) on p73 were studied. On co-transfecting p73α with either p53 mutant and a p53-responsive reporter, both R175H and R248W mutants reduced the transcriptional activity of p73α.[70]

Two other tumour-derived p53 mutants (p53His175 and p53Gly281) associated *in vitro* and *in vivo* with p73α, β, γ, and

δ Were investigated. This association arises under physiological conditions, verified in T47D and SKBR3 breast cancer cell lines.[77] Nevertheless, some data again indicate that p73 binding to mutant p53 is dependent on the cellular context. In fact in H1299 cells mutant p53 does not bind effectively to p73.[78,79]

Several reports have focused on p53 polymorphism. A polymorphic site at codon 72 in exon 4 encodes either an arginine amino acid (72R) or a proline (72P) residue. This polymorphism is located in a proline-rich region of the p53 protein, required for the growth suppression and apoptosis mediated by p53. The prevalence of the polymorphic alleles differs in different populations.[80] In cancer, usually the arginine allele (72R) is predominant over the proline (72P).[81–83] Interestingly the polymorphism at codon 72 can be relevant for inhibition of p73 function. The 72R forms of mutants 143A and 175H associate with p73 and inhibit p73-dependent target gene up-regulation more efficiently than 72P mutants.[81] Other mutants associate with p73 in both 72R and 72P forms.[84]

In head and neck cancer it appears that p53 polymorphism, combined with mutations, influences the response to chemotherapy by an effect on p73-dependent apoptosis.[85] This could be important for the predicting of genetic factors influencing the outcome of clinical treatments. However, the data in head and neck cancer were not reproduced in the lung cancer cell line H1299, in which the response to several widely used drugs was not dependent on the polymorphic status of p53 mutations.[79] Similarly, a recent study analysing p53 polymorphism at codon 72 in ovarian cancer could not confirm the clinical relevance as regards responsiveness to chemotherapy or survival for 72P p53 or 72R mutants.[86] Additional investigations are needed to assess whether the interaction between wild-type p53 and p73 and polymorphic forms of mutant p53 can be taken as an indicator of clinical response to chemotherapy.

10. Cellular response to chemotherapy linked to TAp73

Very soon after the discovery of p73, it was reported that, unlike p53, it is not induced by DNA damaging agents. It was proposed that this new member of the p53 family had no real role in cellular response to damage.[4] The first indication that p73 can in fact respond to cell stress was associated with modifications of the protein: after γ-irradiation, p73 is phosphorylated by c-Abl and this increases its pro-apoptotic ability.[62] Another study obtained similar results on p73 phosphorylation induced by c-Abl at position 99 after γ-irradiation.[63] The amount of p73 protein in the cell also increased on treating the cells with a DNA damaging agent such as cisplatin[87] (Fig. 5).

Phosphorylation of p73 is not the only post-translational modification involved in the cellular response to treatment. Doxorubicin induces acetylation of lysine residues at positions 321, 327, and 331 of p73, mediated by the acetyltransferase p300 (Fig. 5). Inhibiting this enzymatic activity hampers apoptosis in a $p53^{-/-}$ background. In addition, a non-acetylatable form of p73 was defective in activating transcription of the pro-apoptotic p53AIP1 gene.[88] Our laboratory found p73 accumulated after doxorubicin treatment in the neuroblastoma cell line SH-SY5Y and this increase was, at least in part, at transcriptional level.[47] At the same time, one study showed that in striking contrast to the alterations in p53, there was a decline in p73 at both the protein and mRNA levels in several cell lines treated with doxorubicin.[89] Very high concentrations of doxorubicin can induce degradation-mediated down-regulation of p73, whereas up-regulation is seen at more physiological concentrations.

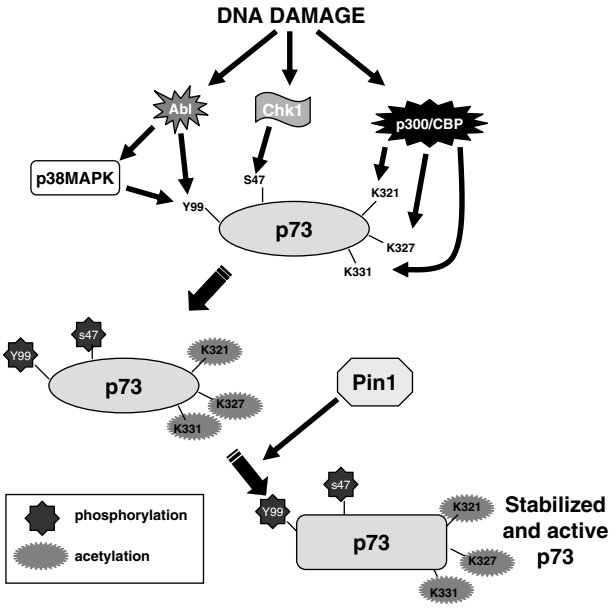

Fig. 5 – Activation mechanisms of p73 after DNA damage.

The checkpoint kinase 1 (Chk1) is an essential component of the DNA damage checkpoint. A recent study provides evidence that p73α is a target of Chk1. Endogenous p73α is serine phosphorylated by endogenous Chk1 upon DNA damage, which is a mechanism required for the apoptotic-inducing function of p73α. In line with this, endogenous p73α interacts with Chk1 and is phosphorylated by Chk1 at serine 47 *in vitro* and *in vivo*. Moreover, mutation of serine 47 abolishes both Chk1-dependent phosphorylation of p73α after DNA damage *in vivo* and the ability of Chk1 to up-regulate the transactivation capacity of p73α[90] (Fig. 5).

Accumulation of TAp73 was shown after a variety of chemotherapeutic agents such as camptothecin, VP16, cisplatin, doxorubicin and taxol.[91] This increase was associated with a cell response since in the presence of a dominant-negative p73 mutant p73DD which blocks the function of p73, resistance to chemotherapy increased in the SW480 cell line. Inhibition of p73 RNA levels through the use of specific siRNA gave the same results. These data linked TAp73 to the chemosensitivity of cancer cells.

Similar data were obtained using hydrogen peroxide, nocodazol, taxol and sorbitol.[92] At the same time, however, our laboratory found data contradicting this conclusion. In the ovarian cancer cell line A2780, hyperexpression of p73α was associated with resistance to cisplatin. These p73 overexpressing cells had increased expression of nucleotide excision repair proteins such as XPD, XPB, and XPG and these changes reduced their sensitivity.[93]

In another study in small cell lung carcinoma, p73α inhibited drug-induced apoptosis, hence response. It was suggested that both the DNA binding domain and the SAM domain were involved. In the same study p73β promoted drug-induced apoptosis.[94] The presence and participation of different spliced forms of p73 might be one reason for the different results. Inhibition of all TAp73 variants cannot distinguish between these forms which may be present at different levels in the tumour.

The p38 MAP kinase pathway participates in this response by enhancing the stability of p73. Transcriptional activation of p73 by c-Abl requires the activity of p38[95] (Fig. 5). p73 can be modified through acetylation and conformational changes catalysed by the prolyl isomerase Pin1 are crucial in this pathway. Lack of Pin1 reduces the stability of p73, hampering its accumulation in response to genotoxic stress. c-Abl enhances the phosphorylation-dependent interaction between Pin1 and p73, and this in turn promotes p73 acetylation by p300. Mantovani and colleagues[96] reported that Pin1 strongly enhances p73-dependent apoptosis in p53-null cell lines, an observation that correlates with the fact that it boosts p73's ability to induce pro-apoptotic target genes, including Bax, Pig3, and p53AIP1. As a consequence, Pin1 appears essential for activation of the apoptotic response mediated by endogenous p73 (Fig. 4).[96]

An intact mismatch repair system also seems important. In cells defective in hMLH1 protein (HCT116) p73 is not induced after cisplatin treatment.[87] Another mismatch repair protein, PMS2, interacts with p73, stabilising it and causing the redistribution of PMS2 to the nuclear compartment. Exposure to cisplatin enhances the association between PMS2 and p73. Stimulation of the p73 pro-apoptotic function by cisplatin also requires PMS2. These results suggest that PMS2 contributes to genome integrity not only through DNA repair but also by enhancing DNA damage-induced apoptosis.[97]

The search for molecules and mechanisms that drive p73 to induce an apoptotic response is very important since p73 is rarely mutated in tumours and can be available for activation.

11. Role of DNp73 isoforms in chemosensitivity of cancer cells

DNp73 isoforms which lack the transactivation domain are theoretically unable to induce gene expression directly and do not cause growth arrest or cell death. At the same time DNp73 isoforms can exert a dominant negative function and block the ability of p53 and p73 to induce apoptosis (Fig. 5). ΔNp73 inhibited p53-dependent transactivation of CD95 and apoptosis in Heb3B cells.[98] Clinical evidence was recently presented that dominant-negative p73 isoforms contribute to drug resistance *in vivo* in ovarian cancers.[86] Another article reported that ΔNp73 stabilises TAp73 proteins but impairs their function due to inhibitory hetero-oligomer formation.[52] Thus the presence of ΔNp73 should give a disadvantage for chemotherapy. The P2 promoter, which controls the expression of ΔNp73, contains a functional p53 site, so ΔNp73 might be induced after damage. This is in contradiction with the apoptotic response of p53 and p73 which could be blocked by ΔNp73 hyperexpression. Nevertheless, ΔNp73 cannot block apoptosis because damage induces rapid degradation of this isoform, allowing apoptosis to occur.[99]

The role of ΔNp73 is complicated and the results are not as linear as for TAp73. Some results on ΔNp73 are quite the opposite of those discussed above. ΔNp73β induced both cell cycle arrest and apoptosis when stably expressed in cancer cells. The 13 unique residues at the N-terminus were required to suppress cell growth.[12] In our laboratory, inducible expression of ΔNp73α in HCT116 cells was not associated with resistance to cisplatin and doxorubicin.[100] These data were confirmed in other cell lines that do not contain endogenous p53.[101] Moreover, cells over-expressing ΔNp73α had no growth advantage *in vivo* when transplanted in nude mice. The mice with ΔNp73α-over-expressing tumours respond to chemotherapy just like those with the same tumour but not expressing ΔNp73α.[100,102] Again, further data in different cell systems are needed to clarify the role of DNp73, if any, in cancer responses to treatment.

12. Conclusion

The discovery of p73 and p63 generated a p53 family and indicated the complexity of the famous oncosupressor. p73 shares great similarity in structure and functions with p53. The recent discovery of p53 isoforms with different functions from the main form predicts future complications in this family. The activities of p73 isoforms can be co-expressed in cells and also oppose those of full-length p73. The main opinion on p73 functions is that in a p53-null background p73 can be responsible for regulating of the cell cycle, apoptosis and cancer cell response to drugs. p73 activates many p53-related genes. It can also induce activities that are not shared by p53 and can even be oncogenic, as exemplified by β-catenin (Fig. 6).

We have discussed the possible inhibition of p53 activity by p73 in some cellular contexts. p73 can induce MDM2, thus helping induce p53 degradation. However, in some cells, cooperation between p53 and p73 was observed. Isoforms of p73 can inhibit the activity of both p53 and p73. p73 can be

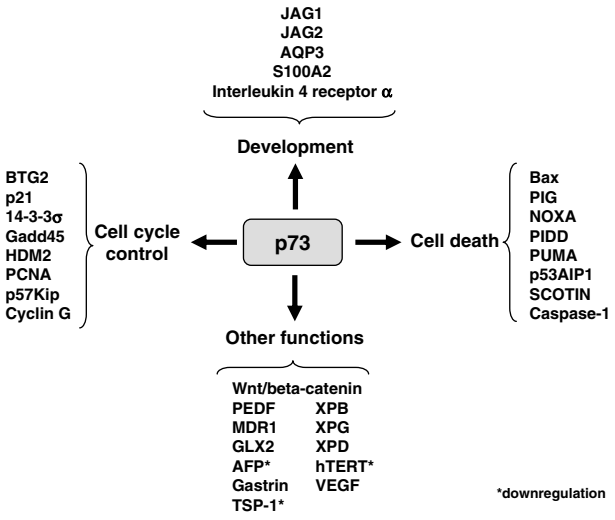

Fig. 6 – Downstream target genes directly regulated by p73.

linked with cell sensitivity, and current studies mainly agree on this point, though it is not valid for every cellular context. In some situations hyperexpression of p73 can cause resistance. p73 co-operates with different transcriptional factors that can either activate or inhibit its activity and this probably helps explain the different behaviour in different cells. DNp73 can be an indicator of poor prognosis in tumours and an inhibitor of cellular response, but some forms of DNp73 can push cells to apoptosis. In some situations DNp73 does not influence cancer sensitivity. It is still debated whether a balance of the levels of TA and DN forms of p73 is needed for tumour suppression.

New activities are coming to light for DN isoforms and they may have unexpected functions, besides simply being regarded as dominant negative regulators of TAp73 and p53. They may inhibit mutant forms of p53 thus helping contrast its oncogenic activity. Specific experiments are needed to tell us whether this is the case.

Therapy with adenovirus expressing p73 has been proposed but the complexity of p73's activity suggests caution in this approach. The role of p73 as another member of the p53 family may prove important but today's contradictory knowledge means further investigations are still needed to understand what can be expected for p73.

In the near future new insights into the roles of the different spliced isoforms of p73 should help in understanding this protein's role in cancer. New data should be forthcoming on the expression of the different isoforms in human tumours and on the correlation between these levels and the clinical outcome. These will probably prove essential for setting up better therapeutic interventions. Many laboratories will probably soon start unravelling the functions of each isoform in terms of expression in tumours.

Conflict of interest statement

None declared.

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