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Chapter: Alterations of p63 and p73 in human cancers

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Summary

p53 and its related genes, p63 and p73 constitute the p53 gene family. While *p53* is the most frequently mutated gene in human tumors, *p63* and *p73* are rarely mutated or deleted in cancers. Many studies have reported p63/p73 overexpression in human cancers while others showed that a loss of p63/p73 is associated with tumor progression and metastasis. Thus, whether p63 or p73 is a tumor suppressor gene or an oncogene has been a matter of debate. This controversy has been attributed to the existence of multiple splicing isoforms with distinct functions; the full-length TA isoform of p63 has structural and functional similarity to wild-type p53, whereas the ΔNp63 acts primarily in dominant-negative fashion against all family members of p53. Differential activities of TA and ΔN isoforms have been shown *in vivo* by creating isoform-specific gene knockout mice. All p53, p63, p73 proteins bind to and activate target genes with p53-response elements; p63 also binds to distinct p63-response elements and regulate expression of specific target genes involved in skin, limb, and craniofacial development. Interestingly, several studies have shown that both p63 and p73 are involved in cellular response to cancer therapy and others have indicated that both of these molecules are required for p53-induced apoptosis, suggesting functional interplay among p53 family proteins. Consistent with these findings, aberrant splicing that result in ΔNp63 or ΔNp73 overexpression are frequently found in human cancers, and is associated with poor clinical outcomes of patients in the latter. Thus immunohistochemical staining of tumor specimen with ΔNp73-specific antibody might have diagnostic values in cancer clinics.

Keywords

p63; p73; splicing; alteration; overexpression; knockout; mouse; cancer

Introduction

The p53 tumor suppressor protein integrates endogenous and exogenous signals to modulate cell fate to stress and cellular environments (Toledo 2006; Menendez 2009; Vousden 2009). Upon DNA damage or other cellular stresses, such as oxidative stress, hypoxia, carcinogen exposure, and oncogene overexpression, p53 becomes activated with increased levels. Then, p53 directs a variety of responses, including DNA repair, cell cycle arrest/senescence, apoptosis, and autophagy depending on the input signal and severity of the damage (Green 2006; Rufini 2013). The specific response depends on whether the damage can be repaired

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or is too serious that death of the cell is required to maintain tissue integrity. The genomic locus for *p53* (*TP53*) is very frequently (~50%) mutated in human cancers, which is associated with therapy resistance and poor prognosis of patients (Xu, 2008; Lai 2012). Since p53 protects humans from damaged and life-threatening cells that may predispose to tumor development, recent research efforts have been made on reconstituting p53 function to effectively treat cancer patients (Frezza & Martins 2012; Senzer 2013).

In the late 1990's, two other p53 family members, p73 and p63 were discovered (Kaghad 1997; Yang 1998). These three proteins, encoded by the *TP53*, *TP63*, and *TP73* genes (*Trp53, Trp63,* and *Trp73* in mice, respectively), are transcription factors that bind directly to DNA as tetramers, interact with other transcription factors and the transcription machinery, and together control the expression of genes involved in all aspects of life. It has now become clear that both p63 and p73 are involved in a broad spectrum of biological activities, such as cell proliferation, apoptosis, development, differentiation, senescence, and aging. In particular, p63 has emerged as a critical player in embryonic development, epithelial stem cell maintenance, and differentiation. Both p63 and p73 express as a variety of protein isoforms that originate from two different promoters and extensive gene splicing at the N- and C-termini (Murray-Zmijewski 2006). Moreover, the p63 and p73 genes encode a sterile alpha motif (SAM) domain at the C-terminus that is not found in p53. This domain is responsible for protein-protein interactions and is found in a diverse range of proteins that are involved in developmental regulation. In this chapter, we discuss the structure, splicing isoforms of p63 and p73 in normal and their distinct functions in tumor suppression/ proliferation. We also explain their possible interaction with Mdm2 and MdmX. Whether these molecules (p63 and p73) are tumor suppressors or oncoproteins have been a hot topic of debate. Gene knockout studies will tell us the answer; since both of the genes have multiple splicing isoforms, we have put special interest on the phenotypes of splicing isoform - specific gene knockout mouse models. Finally, we summarize the mechanisms and frequencies for alterations of these genes in human cancers and their prognostic significance.

Structure of the *p63* and *p73* loci

Both *p63* and *p73* loci (*TP63, TP73*) generate mRNAs that produce multiple protein products resulting from use of distinct promoters and alternative mRNA splicing (Figure 1) (Kaghad 1997; Yang 1998). Transcription of *p63* and *p73* occurs from two promoters: one upstream of exon 1 (P1) and the other located within intron 3 (P2). In both proteins, splicing isoforms transcribed from the P1 promoter have an N-terminal transactivation (TA) domain (i.e., TAp63 and TAp73), which is highly homologous to the TA domain of p53, whereas transcripts generated from the P2 promoter lack the N-terminal TA domain (39 amino acids; called ΔNp63 and ΔNp73, respectively; Figure 1) (Kaghad 1997; Yang 1998). The unique structural differences for p63 and p73 are explained below.

p63

The structure of the genomic locus for p63 is shown in Figure 1, upper panel (Yang 1998). Both mouse and human p63 genes consist of 15 exons spanning around 210kb and 270kb, respectively, on the genome. The human version has been mapped to chromosome 3q27.

The structures for the TAp53 protein, representative p63 isoform proteins are shown in Figure 2 (Murray-Zmijewski 2006). Wild-type TAp53 has an N-terminal transactivation domain (TA) for recruitment of core transcriptional factors, a central DNA-binding domain (DBD) for recognition of promoter sequences, an oligomerization domain (OD) for tetramerization, and a short basic stretch of 30 amino acids for regulation of transcriptional activity (Figure 2, top panel). The *p63* gene encodes two alternatively spliced isoforms (TA, ΔN) with different ATG at the N-terminus with three alternatively-spliced C-terminal isoforms (α, β, γ), generating 6 different splicing isoforms, i.e., TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, ΔNp63γ (Figures). The p63α transcript has all 14 exons while the β transcript lacks exon 13. The γ transcript lacks exons 11–14 by splicing into a unique exon 10′ (Figure 1, top panel). The full-length TA isoform of p63 has structural and functional similarity to wild-type p53, whereas the ΔNp63 acts primarily in dominant-negative fashion against all family members of p53: p53, TAp63 and TAp73. Thus, it is generally assumed that *TAp63* is a tumor suppressor gene while Δ*Np63* is an oncogene. In addition, the C-terminus of p63 (and also p73) contains a sterile alpha motif (SAM) domain and a transcriptional inhibitory domain (TID) (Figure 2). The SAM domains are small protein–protein interaction modules that are found in a wide variety of proteins, ranging from kinases and transcriptional regulators to cell surface receptors (Schultz 1997; Thanos & Bowie 1999). The TID, an unstructured region C-terminal to the SAM domain, was shown to inhibit the transcriptional activity of p63 by interacting with the TA domain (Serber 2002). These two domains are not found in p53 (Figure 2), suggesting unique functions for p63 and p73.

p73

Both mouse and human *p73* genes consist of 15 exons spanning around 80kb on the genome. The structure of the genomic locus for *p73* is shown in Figure 1, lower panel. The human version has been mapped to chromosome 1p36.33. The *p73* gene encodes 4 alternatively spliced isoforms (TA, Δex2, Δex2/3, ΔN) with distinct ATG at the N-terminus and 7 alternatively spliced isoforms at C-terminus (α, β, γ, δ, ε, ζ, and η; Figure 1) (Moll & Slade 2004; Bourdon 2005). In addition, splicing-associated frameshifts yield unique C-terminal sequences for some p63 and p73 isoforms (Courtois 2004; Moll & Slade 2004). This alternative splicing can generate 28 plus one (ΔN′; total 29) different splicing isoforms for p73. Of note, both ΔN and ΔN′ isoforms have unique amino acids at exon 3′ (Figure 1). The difference in the N-terminal region contributes to different protein-protein interactions dependent on the isoform. The p73α transcript has all exons 1–14 while the β transcript lacks exon 13. The γ transcript lacks exon 11, the δ transcript lacks exons 11–13 (Figure 1, lower panel). The ε isoform lacks 11 and 13, ζ lacks exons 11 and 12; η is close to α, but is different at exon 14 (Figure 1). The TAp63γ and TAp73γ isoforms most closely resemble the full-length wild-type TAp53 (Figure 2). In overexpression studies, TAp63γ has been shown to be as potent as p53 in transactivating target gene expression and apoptosis, whereas the most potent transcriptionally active p73 isoform reported is TAp73β (Kaghad 1997; Yang 1998). Since the ΔNp73 acts primarily in dominant-negative fashion against all family members of p53, it is generally accepted that *TAp73* is a tumor suppressor gene while Δ*Np73* is an oncogene.

Unique C-terminal domains and transcriptional targets for p63 and p73

Both p63α and p73α isoforms also contain a protein–protein interaction domain known as SAM (Figure 2). This is a globular domain composed of four α-helices and a small 310 helix. Although this motif is often found to mediate homodimerization with developmentally regulated proteins, the SAM domain does not contribute to homodimerization in p63 and p73 (Chi 1999). The SAM domains also appear to possess the ability to bind RNA. The post-SAM region known as the transactivational inhibitory domain (TID) has been identified in p63α and p73α isoforms (Serber 2002; Figure 2). This region consisting of ~70 amino acids, which is absent in p53, has been proposed to inhibit transcription of both TAp63α and TAp73α through inter- or intra-molecular association with the TA domain (Serber 2002). Indeed, both of these proteins show decreased potency in transactivation and apoptosis induction as compared to other TA isoforms, and deletion of this region restored transactivating potential for both TAp73α and TAp63α (De Laurenzi 1998, Serber 2002).

Since both p63 and p73 share strong structural, biochemical and biological homologies to p53, they bind specifically to conventional p53 response elements (p53RE: RRRCWWGYYY) and transactivate target genes such as *p21*^{Cip1}, *MDM2*, and *BAX*. In spite of their structural similarities between p53 and p63, the latter functions are greatly different from those of p53. The most striking difference is the apparent involvement of p63 in skin and limb development (Mills 1999; the details of phenotypes will be explained later). Global *p63* knockout mice that lack all splicing isoforms exhibit skin and limb defects as well as craniofacial abnormalities, but are not tumor prone. This is in contrast to *p53* knockout mice that develop normally, but are prone to develop various cancers from an early age, esp. thymic lymphomas and hemangiosarcomas (Donehower Nature 1992; Jacks 1994). In humans, germ line mutations of *p53* cause Li-Fraumeni syndrome, in which affected individuals are very prone to cancer development (Malkin 1990, 1993; Strong 1992). These differences may be due to the differential regulation of target genes by p53 and p63. The p53 and p63 proteins can bind to two or more tandem repeats of RRRCWWGYYY (p53RE) or some other motifs and subsequently activate target gene expression. By using oligonucleotide expression microarray analysis and analyzing the promoters of p63-induced genes, Osada *et al*. (2005) identified novel p63-specific response elements (p63REs) in the promoter regions of *EVPL* and *SMARCD3*. These p63REs exhibit characteristic differences from the canonical p53RE (RRRCWWGYYY) in both the core-binding element (CWWG) as well as the RRR and/or YYY sequences (Osada 2005). Their data indicate that p53 preferentially activates and binds to the RRRCATGYYY sequence, whereas p63 preferentially activates RRRCGTGYYY. Whereas EVPL protein is highly expressed in epithelial cells of the skin and pharynx in the *p63*$^{+/+}$ mouse, it is undetectable in these tissues in the *p63*$^{-/-}$ mouse. Thus p63 can regulate expression of specific target genes such as those involved in skin, limb, and craniofacial development by preferentially activating distinct p63-specific response elements (Osada 2005). Until now, a number of genes have been reported to be targets of p63 and p73, such as *REDD1* (regulation of reactive oxygen species), *JAG1*/*JAG2* (Notch signaling), *IL4R*, *ΔNp73*, and *AQP3* (glycerol and water transporter) (Ellisen 2002; Sasaki 2002; Osada 2005). Among these, *ΔNp73* is a splicing variant from the *p73* locus, suggesting its autoregulation (Nakagawa 2002). In Notch signaling, Sasaki *et al*. found that the genes encoding ligands for the Notch receptors

(*JAG1/2*), are up-regulated by p63 and p73 but not by p53 (Sasaki 2002). They identified a p63-binding site in the second intron of the *JAG1* gene, which could directly interact with p63 *in vivo* as demonstrated by chromatin immunoprecipitation. They also found a target of Notch signaling; HES-1 was up-regulated in Jurkat cells with high expression of Notch1 when co-cultured with p63-transfected cells, suggesting that p63 can trigger the Notch signaling pathway in neighboring cells. This suggests a potential molecular mechanism for the involvement of p63 in normal development (Sasaki 2002). Recently it was reported that BRCA1 activates the Notch pathway in breast cells by transcriptional upregulation of Notch ligands and receptors (Buckley 2013). They demonstrated that BRCA1 was localized to an intronic enhancer within the *JAG1* gene, an event requiring ΔNp63. This BRCA1/ΔNp63-mediated induction of *JAG1* must play important roles in the regulation of breast stem/precursor cells since knockdown of these proteins resulted in increased tumorsphere growth and increased activity of stem cell markers (Buckley 2013). Thus, BRCA1/ΔNp63-mediated transactivation of Notch signaling is a key event in the normal differentiation process in breast tissue.

Regulation of p63 and p73 by Mdm2

The interactions of p53 with Mdm2 and Mdmx, mediated via the TA domain of p53 have been well-documented (Lin 1994; Kussie 1996; Deb 2003; Popowicz 2008). The physiological importance of the regulation of p53 by the Mdm2 and Mdmx ubiquitin ligases as well as the role of its aberrant regulation in tumors has also been reported (Kubbutat 1997; Haupt 1997; Danovi 2004; Linke 2008; Okamoto 2009). Unlike p53, which protects genomic stability, the two homologous proteins of the same family, p63 and p73, regulate developmental processes as described in this chapter.

Since all three p53 family proteins have homologous TA domains, it was speculated that p63 and p73 may be regulated by Mdm in a similar manner as has been reported for p53. The ability of Mdm2 and Mdmx to bind to p73 has been well-documented (Ongkeko 1999) and Zdzalik *et al.* (2010) provided a detailed kinetic characterization of this interaction. The interaction of Mdm with p63 has also been studied previously, but the results were controversial due to lower affinity for such an interaction (Kojima 2001; Little 2001; Wang 2001; Calabro 2002). In fact, Zdzalik *et al.* (2010) showed that both Mdm2 and Mdmx form complexes with the p63 TA domain, however the interactions were weaker than those determined for p53 or p73. The interaction of the p63 TA domain is specific and mechanistically similar to that of the p53 TA domain since the p63(Ala) mutant peptide showed no activity in the assays performed. Although the interactions of p73 with Mdm2 and Mdmx have also been studied previously, only the affinity of p73 for Mdm2 has been reported (Schon 2002; Burge 2009). The interaction between p63 and Mdm2 was one order of magnitude weaker than those of Mdm with p53 and p73. Conversely, the affinities of both Mdm2 and Mdmx for p73 were of the same order of magnitude as those for p53, which justifies the conclusion that these proteins truly interact in cells, as has previously been suggested in other studies (Ongkeko 1999, Wang 2001). The weaker interactions of both Mdm2 and Mdmx with p63 explain the inconsistent results reported by different groups on the interactions of those proteins. Clearly, at sufficiently high concentrations, these proteins will form a stable complex, but whether such concentrations are ever encountered under

physiological conditions in cells remains a very intriguing question for future studies (Zdzalik 2010). It is also noteworthy that although the affinities of p53 for Mdm2 and Mdmx are similar, both p63 and p73 interact more strongly with Mdmx. Therefore, Mdmx, but not Mdm2, may have a stronger impact on the regulation of intracellular p63 and p73.

Constitutional, all splicing isoforms' knockout mice for p63, p73

Mills *et al*. (1999) reported the phenotypes of *p63*-deficient mice (all splicing isoforms). *p63*-null mice were born alive but had striking developmental defects. Their limbs were absent or truncated, defects that were caused by a failure of the apical ectodermal ridge differentiation. The skin of *p63*-null mice did not progress through an early developmental stage lacking stratification with no differentiation markers. Hair follicles, teeth and mammary glands were absent in *p63*-deficient mice. Thus, in contrast to p53, p63 is essential for several aspects of ectodermal differentiation during embryogenesis. Keyes *et al*. (2006) studied spontaneous and chemically-induced tumor development using $p63^{+/-}$ mice since $p63^{-/-}$ mice had serious developmental defects, and thus were not suitable for *in vivo* tumor development studies. They found that $p63^{+/-}$ mice were not tumor prone and mice heterozygous for both p63 and p53 had fewer tumors than $p53^{+/-}$ mice. Furthermore, p63 expression was maintained in carcinomas. These findings demonstrate that p63 plays a markedly different biological role in cancer than p53.

Mice deficient for all *p73* splicing isoforms also exhibited profound developmental and immunological defects, including hippocampal dysgenesis, hydrocephalus, chronic infections, and inflammation, as well as abnormalities in pheromone sensory pathways (Yang 2000). It should be noted that mice lacking *p73* showed no increased susceptibility to spontaneous tumorigenesis, in contrast to *p53*-deficient mice (Donehower 1992, Jacks 1994). They speculated that potentially dominant-negative, p73 variants were the predominant expression products of this gene in developing and adult tissues, explaining the mechanistic basis of the hippocampal dysgenesis and the loss of pheromone responses in *p73*-null mice. In conclusion, p73 plays unique roles in neurogenesis, sensory pathways, and homeostatic control, but its role in tumor suppression is not clear from the global gene deletion model.

Flores *et al.* (2002) explored the combined role of p63 and p73 in DNA damage-induced apoptosis. The combined absence of *p63* and *p73* severely impaired the induction of p53-dependent apoptosis in response to DNA damage in E1A-expressing cells and in the developing central nervous system in mice although the p53 locus remained intact. This was explained by the inability of p53 to bind the promoters of apoptosis-associated target genes and to upregulate their transcription in $p63^{-/-}; p73^{-/-}$; E1A(+) cells and the developing central nervous system (Flores, 2002; Taneja 2011).

Splicing isoform-specific knockout mouse models for p63, p73

p63

The roles of *p63* in tumor suppression have been a hot topic of debate. The most intriguing question is whether p63 is a tumor suppressor gene or an oncogene? Many studies have

shown p63 overexpression in human cancers (Massion 2003; discussed later in this chapter), while others demonstrate that a loss of p63 is associated with tumor progression and metastasis (Flores 2007). This controversy has been attributed to the existence of multiple splicing isoforms with distinct functions; the full-length TA isoform of p63 has structural and functional similarity to p53 (Figure 2), whereas the ΔNp63 protein acts primarily in dominant-negative fashion against all family members of p53. To study splicing isoform–specific differences of *p63* functions *in vivo,* Su *et al.* (2009) developed a *TAp63* conditional knockout mouse and used it to delete *TAp63* in the germline (*TAp63*$^{-/-}$; using *Zp3-cre* or *Protamine-cre*) or in K14-expressing cells in the basal layer of the epidermis (epidermis-specific *TAp63* deletion; using *K14cre+*). *TAp63*$^{-/-}$ mice aged prematurely and developed blisters, skin ulcerations, senescence of hair follicle-associated dermal and epidermal cells, and decreased hair morphogenesis, indicating that TAp63 serves to maintain adult skin stem cells by causing cellular senescence and genomic stability, thereby preventing premature tissue aging (Su 2009). The same group followed spontaneous tumor development in *TAp63*$^{-/-}$, *TAp63*$^{+/-}$ and wild-type mice for 2.5 years and found that both *TAp63*$^{+/-}$ mice and *TAp63*$^{-/-}$ mice developed carcinomas and sarcomas with significantly shorter lifespan than the wild-type cohort. Consistent with this finding, tumors from *TAp63*$^{+/-}$ mice retained the wild-type allele of *TAp63* suggesting that *TAp63* is haplo-insufficient for tumor suppression. Both *TAp63*$^{+/-}$ and *TAp63*$^{-/-}$ mice developed highly metastatic tumors, and 10% of these metastases were found in the brain, a rare finding in endogenous mouse tumor models. Although equivalent numbers of carcinomas metastasized in the *TAp63*$^{-/-}$ and *TAp63*$^{+/-}$ mice, a greater number of sarcomas metastasized in *TAp63*$^{+/-}$ mice than in *TAp63*$^{-/-}$ mice, indicating that heterozygous loss for *TAp63* rather than homozygous loss results in a more severe phenotype.

Keyes *et al.* (2011) observed that ΔNp63α overexpression in mouse embryonic fibroblasts (MEFs) bypassed Ras-mediated senescence and drove tumorigenesis *in vivo*. They identified chromatin-remodeling protein Leeh as a novel target for ΔN63α, which was an essential mediator of senescence bypass. This bypass of senescence by ΔNp63α promoted stem cell-like proliferation and maintained the survival of keratin 15-positive cells. Thus, ΔNp63α is a novel oncogene that cooperates with Ras to promote tumor development by initiating stem cell proliferation. By contrast, overexpression of TAp63 forms in *p53*$^{-/-}$ MEFs increased senescence and reduced tumor development *in vivo*, consistent with a p53-independent effect of TAp63 (Guo 2009).

The TAp63 and ΔNp63 isoforms have special effects in epidermal tissue differentiation (Aberdam 2007). In murine embryonic stem cells, ΔNp63, but not TAp63, is highly expressed in epidermis and is critical for the expression of the cytokeratins K5 and K14, two markers of keratinocyte differentiation, indicating that only ΔNp63 is required for the commitment of ectodermal into epidermal cells (Medawar 2008; Shalom-Feuerstein 2011). In summary, p63 and its splicing variants play specific roles in epidermal commitment, cell proliferation, and senescence bypass; alterations of this intricate balance contribute to tumor development.

p73

Mice with a complete deficiency of *p73* exhibited severe neurological and immunological defects due to the absence of all *TAp73* and *ΔNp73* isoforms as described in the previous section. To study mice deficient for specific p73 protein isoforms, Tak Mak's group created *p73* isoform-specific knockout mice (Tomasini 2008; Wilhlem 2010). Tomasini *et al.* (2008) created mice in which exons encoding the *TAp73* isoforms were specifically deleted at exons 2/3 to establish a *TAp73*-deficient ($TAp73^{-/-}$) mice. Mice specifically lacking in *TAp73* isoforms showed a phenotype intermediate between the phenotypes of $p73^{-/-}$ and $p53^{-/-}$ mice with respect to the incidence of spontaneous and carcinogen-induced tumors, infertility, and aging, as well as hippocampal dysgenesis. In addition, cells from $TAp73^{-/-}$ mice showed genomic instability associated with enhanced aneuploidy, which could account for the increased incidence of spontaneous tumors in these animals. Hence, *TAp73* isoforms exert tumor-suppressive functions indicating an emerging role for *Trp73* in the maintenance of genomic stability. Wilhlem *et al.* (2010) generated mice that were selectively deficient for the *ΔNp73* isoform by depleting *ΔN* form-specific exon 3′ ($\Delta Np73^{-/-}$). These mice were viable and fertile, but showed signs of neurodegeneration. Cells from $\Delta Np73^{-/-}$ mice were sensitive to DNA-damaging agents and showed an increase in p53-dependent apoptosis. They found that the ΔNp73 protein localized directly to the site of DNA damage, interacted with the DNA damage sensor protein 53BP1, and inhibited ATM activation and subsequent p53 phosphorylation. This finding may explain why human tumors with high levels of ΔNp73 expression showed resistance to chemotherapy.

In summary, these studies show that TAp63 and TAp73 proteins have specific roles in preventing tumor development *in vivo*. Conversely the ΔN forms act as oncogenes by preventing senescence and maintaining progenitor cell status. When overexpressed, both TAp63 and TAp73 proteins transactivate subsets of known p53 target genes involved in cell-cycle arrest and apoptosis, such as $p21^{Cip1}$ and *Bbc3* (Zhu 1998; Dohn 2001; Melino 2002, 2011). Of note, both TAp63 and TAp73 also regulate distinct sets of genes that are not transcriptional targets for p53 through unique p63RE as described in the previous section. In contrast, ΔNp63 and ΔNp73 proteins have been shown to function in part as dominant-negative inhibitors of the p53 family, leading to the hypothesis that these isoforms may exhibit proto-oncogenic function. ΔN isoforms inhibit the function of TA forms through 1) direct competition for DNA-binding sites and 2) formation hetero-oligomeric complexes with TAp63/TAp73, and less strongly with p53 (Yang 1998; Davidson 1999; Chi 1999; Grob 2001; Stiewe 2002; Chan 2004). Interestingly, expression of the ΔNp73 is strongly up-regulated by TAp73 and p53, thus creating a feedback loop that tightly regulates the function of TAp73 and more importantly, of p53 (Grob 2001).

Aberrant expression, altered splicing, and mutations of p63 and p73 in human cancer

Alterations of p63 isoforms in human cancers

Both p63 and p73 were initially hypothesized to function as tumor suppressors based on their homology to p53. However, accumulating evidence shows that mutation of either of these genes in human cancer is quite rare (Yoshikawa 1999; Ichimiya 1999), indicating that

they are not classical tumor suppressor genes like *p53* or *RB* that meet the Knudson's two-hit hypothesis (Knudson 1971). Although there have been numerous studies on p63 expression in human cancers, loss of heterozygosity (LOH) of the *p63* locus has not been studied extensively in human malignancies (Zaika & El-Rifai 2006), possibly because the genomic locus 3q27–28 is not the site of frequent gene deletion in cancer. Conversely, decreased p63 expression is a common feature of high-grade invasive urothelial carcinomas and associates with reduced β-catenin. Both ΔNp63 and TAp63 are frequently downregulated in bladder cancer and this reduction correlates with a poor prognosis (Park 2000). The majority of prostate cancers show loss of p63, but it is overexpressed in some poorly differentiated tumors and correlates with a poor prognosis (Grismazio 2008). In addition, loss of p63 results in enhanced metastasis in prostate cancer (Tuccu 2012). Koga *et al.* (2003) studied the expression of p63, β-catenin, and uroplakin III by immunohistochemistry in high-grade invasive bladder carcinomas. Lower p63 expression was significantly associated with higher TNM stage, lymph-node metastasis, reduced β-catenin expression, and thus with poor prognosis. Impaired p63 expression was associated with biological aggressiveness of high-grade invasive urothelial carcinomas. Moreover, loss of p63 expression was a pre-requisite for uroplakin III expression. Their data suggested that p63 plays critical roles in the prevention of tumor progression and biochemical terminal differentiation of urothelial neoplasms (Koga 2003).

Oral lichen planus (OLP) is a relatively common chronic disease of the oral mucosa for which the etiology or pathogenesis is not fully understood. Sniezek *et al.* (2002, 2004) showed decreased expression of p63 in OLP compared to normal mucosa, a decrease they suggested could explain the hyper-differentiation, or pro-differentiation, seen in OLP. Consistent with these findings, another group reported downregulation of p63 (Ebrahimi 2006) in OLP and GVHD associated with oral inflammation.

The *p63* gene maps to chromosome 3q27–28, a region frequently amplified in squamous cell carcinomas (Hibi 2000; DiComco 2002; Massion 2003; Zaika & El-Rifai 2006; Brunelli 2012). Most squamous cell carcinomas retain p63 expression, where it is often overexpressed (Massion 2003; DeYoung 2006; King 2007). Although some controversy exists as to whether p63 is the targeted gene driving amplification of this locus, several groups have reported increased p63 mRNA levels that correlate with an increase in *p63* gene copy number in squamous cell carcinomas of the lung, head, and neck (HNSCCs) (Hibi 2000; Tonon 2005). In other cases, overexpression of p63 appears to be independent of genomic DNA amplification of the locus (Redon 2001). In esophageal carcinomas, amplification of the *p63* gene was reported in ~20% of squamous cell carcinomas and 10% of adenocarcinomas (Zaika & El-Rifai 2006). Given that the total frequency of tumors in which p63 is upregulated is much higher (>50%), gene amplification is unlikely to be the main mechanism underlying the increased levels of p63. Rather, transcriptional or post-transcriptional changes are involved. Multiple studies have shown that p63 overexpression occurs in up to 80% of primary HNSCCs and also in other squamous cell carcinomas, including those in the lung, nasopharynx, and cervix (Wang 2001; Hu 2002; Weber 2002; Massion 2003). By the use of isoform-specific antibodies, Nylander *et al.* (2002) mapped expression of the different p63 isoforms within normal oral mucosa and HNSCCs, showing increased expression of p63, mainly the ΔNp63 isoforms, in tumors compared to normal

mucosa. They indicated specific roles for the individual isoforms in cell differentiation and neoplasia (Nylander 2002). In invasive breast cancer, the frequency of p63 expression varies, ranging from 0 to 30% (Wang 2002; Reis-Filho 2003; Koker & Kleer 2004). It is now considered that p63 is expressed in at least a subset of breast tumors that are known to have a basal epithelial phenotype (Livasy 2006).

TAp63 vs. ΔNp63 in cancer

In esophageal carcinomas, p63 isoforms are upregulated not only in carcinomas, but also in squamous dysplasias (Zaika & El-Rifai 2006). Although early studies for the detection of p63 did not differentiate among different isoforms, recent studies used isoform-specific RT-PCR coupled with Western blot analysis to quantitatively demonstrate that ΔNp63α is the predominant p63 isoform expressed in squamous cell carcinomas. Using such an approach, it has been reported that tumor-suppressive TAp63 overexpression is rare in HNSCC, and that *ΔNp63* mRNA expression was at least 100-fold more abundant than *TAp63* mRNA in all cases (Deyoung 2006; Rocco 2006). These findings are consistent with the inability of many investigators to detect TAp63 protein isoforms by Western blot analysis in either primary keratinocytes or HNSCC cells. ΔNp63 is the predominant variant that is found in HNSCCs; however, in Barrett's esophagus, a disorder in which the stratified epithelium is replaced by a simple columnar epithelium that consists of mucosecretory cells, the *p63* gene expression is not highly prominent (Zaika & El-Rifai 2006).

Tumors often have simultaneous transcriptional upregulation of both *TAp63* and Δ*Np63* isoforms, with ΔNp63 being predominant at protein levels (Massion 2003; DeYoung 2006). This would represent the anti-apoptotic and proliferative effects of ΔNp63 as described in the previous section. Moreover, it was reported that ΔNp63*α* expression directly correlates with a poor response to cisplatin in HNSCC (Zangen 2005). In pancreatic cancer, Danilov *et al.* (2011) showed that ΔNp63α enhanced the oncogenic potential of tumor cells through trans-activation of *EGFR* and *14-3-3σ*. Leong *et al.* (2007) reported that the p63/p73 network mediates chemosensitivity to cisplatin in a subset of primary breast cancers. Thus, p63 is involved in chemosensitivity of multiple types of tumors. In HNSCC, DNA damage by chemotherapy caused a decrease in ΔNp63-mediated transcriptional repression by blocking p63-responsive elements or sequestering TAp63 in less active hetero-tetramers, together with increased expression of p73, thus allowing TAp73-mediated cell death (Rocco 2006). Together, these reports indicate that it is not only the levels of individual p53 family members, but rather the ratio between TA (transcriptionally active, having tumor-suppressor functions) and ΔN (acting as dominant-negative over the TA isoforms, showing oncogenic properties) isoforms that determines the biological outcome.

In lung cancer, amplification of chromosomal region 3q26-3qter is frequently found in tumors. Massion *et al.* (2003) analyzed *p63* gene copy number and expression by immunohistochemistry in tissue microarrays of >200 non-small cell lung cancers (NSCLCs) and correlated them with survival. The *p63* genomic locus was amplified in 88% of squamous cell carcinomas, but only in 11% of adenocarcinomas of the lung, indicating clear association of gene amplification with squamous cell lung cancer. The major splicing variant of p63 expressed was ΔNp63α. Furthermore, *p63* genomic amplification and protein

staining was associated with better survival. They found a significant increase in *p63* copy number in pre-invasive lesions graded severe dysplasia or higher. Thus, there is early and frequent genomic amplification of *p63* in the development of squamous carcinoma of the lung and patients with NSCLC showing amplification and overexpression of p63 had prolonged survival (Massion 2003). However, two other groups have failed to demonstrate the favorable prognostic value of p63 in lung cancer. Iwata *et al.* (2005) reported a lack of prognostic significance regarding ΔNp63 immunoreactivity in lung cancer. Uramoto *et al.* (2006) showed that the expressions of ΔNp63 in lung cancer did not significantly affect survival while patients with a positive ΔNp73 expression had a poorer prognosis in comparison to the negative group. The differential prognostic values of p63 in these, Massion's and two other studies, can be attributed to the fact that the former study focused on gene copy number of *p63* and immunohistochemical staining of p63 (all splicing isoforms) in squamous cell lung cancer while the latter two groups studied the expression of the ΔNp63 protein and survival of non-small cell lung cancer (in Uramoto's study; squamous cell carcinoma only in Iwata's study).

ΔNp63α can act as a transcriptional repressor, but the link between the transcriptional functions of p63 and its biological role is still unclear. Barbieri *et al.* (2006) depleted endogenous p63 by shRNA to investigate the transcriptional programs controlled by p63. Disruption of p63 in squamous cell carcinoma cell lines resulted in down-regulation of transcripts specifically expressed in squamous tissues and a significant alteration of keratinocyte differentiation. They found that depletion of p63 led to up-regulation of markers of non-epithelial tissues (mesenchyme and neural tissue) in squamous cell carcinomas, which were associated with increased capacity for invasion and metastasis in tumors. Furthermore, loss of p63 expression was accompanied by a shift toward mesenchymal morphology and an increase in motility in primary keratinocytes and squamous cell lines (Barbieri 2006). Thus, loss of endogenous p63 results in up-regulation of genes associated with invasion and metastasis, and predisposes to a loss of epithelial markers and acquisition of mesenchymal characteristics. Although the squamous cell carcinoma cell lines they analyzed expressed predominantly ΔNp63, the interpretation of their experimental results is controversial since their shRNA depleted both *TAp63* and *ΔNp63* at the same time. *p63* isoform-specific shRNA should be used to define the roles of each isoform in cell growth, differentiation, invasion and metastasis.

Regulation of gene expression by ΔNp63

Although ΔNp63 lacks the amino-terminal transactivation domain consisting of 39 amino acids that is present in TAp63, ΔNp63 still activates a group of genes that includes, but is not restricted to genes regulated by p53 (Dohn 2001). Helton *et al.* showed that all NH2-terminally deleted p63 isoforms still retain a potential in transactivation and growth suppression (2006). Interestingly, they showed that ΔNp63β possessed a remarkable ability to suppress cell proliferation and transactivate target genes, which is consistently higher than that seen with ΔNp63α. They showed that an intact DNA-binding domain is required for ΔNp63 function. In addition, they found that the novel transactivation domain for the ΔNp63 variant was composed of the 14 unique ΔN residues along with the adjacent region, including a PXXP motif (Helton 2006). They also showed that a PPXY motif shared by

ΔNp63α and ΔNp63β was required for optimal transactivation of target gene promoters (Helton 2006). Very recently, Ceraldo *et al.* (2013) identified a novel p63 transcriptional target, caspase-1. Caspase-1 is pro-inflammatory caspase, which functions in tumor suppression. They showed that both p63 isoforms (TAp63, ΔNp63) increased caspase-1 expression through physical binding to its promoter. Consistently they also identified a direct correlation between p63 and caspase-1 expression in human cancer data sets. Functional interaction between p63 and caspase-1 represented a predictor of longer survival in human cancers. Together, in addition to dominant-negative effects of ΔNp63 on TA isoforms of p53 family proteins, regulation of gene expression by ΔNp63 variants should be re-evaluated from the viewpoint of tumor suppression.

Alterations of p73 isoforms in human cancers

The *p73* gene has been speculated to be classical tumor suppressor genes like *p53* when the cDNAs were cloned (Kaghad 1997). In gastrointestinal tumors, LOH for *p73* has been reported in 10–40% of the cases (Zaika & El-Rifai 2006) although LOH for *p63* has not been reported in cancers. Despite these expectations, subsequent studies have demonstrated that the *TP73* locus was not the hot spot of gene deletion in cancers. Rather, studies of multiple tumor types have shown that p73 splicing variants are overexpressed, but not mutated or deleted in human malignancies (Moll & Slade 2004).

To investigate the role of the *p73* gene in human carcinogenesis, Han *et al.* (1999) studied genetic alterations of this gene by analyzing the entire coding exons as well as their surrounding exon-intron boundaries by PCR-CCSP and direct sequencing with primary samples from breast, colorectal, gastric cancers, neuroblastomas, and also with lung and pancreatic cancer cell lines since they are known to have frequent LOH in the 1p region. However, of the 185 cases, somatic missense mutation of glutamine from arginine at codon 269 was found in only one breast cancer. Monoallelic expression of p73 was observed in pancreatic cancer cell lines. Nomoto *et al.* (1998) analyzed 61 primary lung cancer samples of the *p73* locus at 1p36.33 by PCR-SSCP and Southern blotting. Although allelic loss at the 1p36.33 locus was observed in 42% of cases, somatic mutations of the *p73* gene were not observed in their samples, suggesting the presence of an as yet to be determined tumor suppressor gene at the locus. In summary, inactivation of the *p73* gene is very rare even in cancers involving chromosome 1p (Han 1999).

TAp73 vs. ΔTAp73 in human cancers

Overexpression of p73 mRNA and/or protein relative to neighbor normal tissues has been reported in a variety of tumors, such as neuroblastoma, glioma, ependymoma, breast, lung, colon, stomach, liver, ovarian, bladder, cholangiocellular carcinomas, and myelogenous leukemias (Moll & Slade 2004). Concin *et al.* (2004) studied the expression profile of all N-terminal isoforms, distinguishing between TAp73 and ΔTAp73 (ΔNp73, ΔN′p73, Δex2p73, and Δex2/3p73) (Figure 2). Ovarian cancers almost universally overexpressed ΔN′p73 compared with normal tissues (95% of cancers). About one-third of tumors also exhibited concomitant up-regulation of TAp73, whereas only a small subgroup of tumors overexpressed ΔNp73 (Concin 2004). Thus, deregulation of the E2F-responsive P1 promoter, rather than the P2 promoter, is mainly responsible for the production of ΔTAp73

in ovarian cancer. A trend was found for better overall survival in patients with low expression of ΔN′p73/ΔNp73, compared with those with high expression. Cancers with wild-type p53 showed significantly higher deregulation of ΔNp73, ΔN′p73, and Δex2/3p73 (transdominant p73) than p53 mutant cancers. Thus, overexpression of transdominant p73 isoforms can function as epigenetic inhibitors of p53 *in vivo*, thereby alleviating selection pressure for *p5*3 mutations in ovarian cancer (Concin 2004).

Dominguez *et al*. (2006) analyzed 113 colon and 60 breast cancer patients' primary samples and reported the association of ΔTAp73 variants and advanced pathologic stage, lymph node metastasis, vascular invasion, presence of polyps, and tumor localization. Overexpression of TP73 variants in tumor tissues indicates that they may be involved in carcinogenesis. The association between upregulation of ΔTAp73 isoforms and poor prognosis suggests that they may be of practical clinical prognostic value. Faridoni-Laurens *et al*. (2008) analyzed the expression of TAp73 and ΔTAp73 in HNSCC and compared them to the p53 status. They found that all of the p73 isoforms were upregulated in comparison to those in normal adjacent tissue. Although p73 belongs to the gene family of p53, *p53* mutations and *p73* transcript alterations were not mutually exclusive. All of the HNSCC specimens studied had at least one p53 mutation and/or one *ΔTAp73* transcript alteration. Although both the ΔNp73 and the TAp73 transcripts were upregulated in HNSCC, the predominant protein in the cancers expressed was ΔNp73. Furthermore, a trend was found for better overall survival in patients with a low expression of ΔNp73. Thus deregulation of both the p53 and the p73 pathways plays an important role in inducing HNSCC (Faridoni-Laurens 2008).

By using specific polyclonal ΔNp73 antiserum against the exon 3′-specific peptide for p73, ΔNp73 and ΔN′p73 expressions were studied in paraffin-embedded tumor samples from 132 lung cancer samples (Uramoto 2004, 2006). The ΔN/ΔN′p73 protein was detected mainly in the cytoplasm of tumor cells in 77 of 132 patients (58.3%) with lung cancer. Importantly, lung cancer patients with positive ΔN/ΔN′p73 expression had a poorer clinical outcomes than those with negative expression. In addition, multivariate analysis of the clinicopathological characteristics of lung cancer indicated that positive expression of ΔN/ΔN′p73 was a significant independent factor for predicting poor prognosis ($P < 0.0001$, risk ratio = 3.39). Thus, expression of ΔN/ΔN′p73 will be a useful marker for predicting poor prognosis of patients who undergo resection of lung cancer. Consistent with these findings, overexpression of the N-terminal splice variants (Δex2p73, Δex2-3p73), but not TAp73, was shown to be associated with a poor prognosis in low-grade gliomas (Wager 2006), which should be helpful in decision-making in clinics.

The truncated oncogenic isoform Δex2p73 is expressed in hepatocellular carcinomas (HCC); however, the underlying mechanisms regulating this process are unknown. Castillo *et al*. (2009) used human normal and diseased liver tissue samples to examine the association between activation of epidermal growth factor receptor (EGFR) by its ligand amphiregulin (AR) and the alternative splicing of *p73* pre-mRNA into the tumorigenic isoform Δex2p73, via c-Jun N-terminal-kinase-1-mediated signaling. Δex2p73 was expressed in a subset of premalignant cirrhotic livers and in otherwise healthy livers that harbored a primary tumor, as well as in HCC tissues. Δex2p73 expression was correlated with that of the EGFR ligand AR, previously shown to have a role in hepatocarcinogenesis. Autocrine activation of the

EGFR by AR triggered c-Jun N-terminal kinase-1 activity and inhibited the expression of the splicing regulator Slu7, leading to the accumulation of Δex2p73 transcripts in HCC cells. Their study provided a mechanism for the generation of pro-tumorigenic Δex2p73 during liver tumorigenesis via activation of EGFR signaling by AR and c-Jun N-terminal kinase-1 activity, leading to inhibition of the splicing regulator Slu7 (Castillo 2009). This is a unique report that showed the specific role of a particular splicing factor in aberrant *p73* splicing.

The molecular mechanisms underlying overexpression of ΔNp63 or ΔNp73 in cancers in comparison to normal tissues need further investigation. Methylation-mediated silencing of the P1 promoter for *TAp73* was reported in lymphoblastic leukemias and Burkitt's lymphomas (Corn 1999; Kawano 1999). These findings indicate that either ΔNp63 or ΔNp73 overexpression or TAp73 promoter silencing is required to inactivate the tumor-suppressive activity of TAp73. Although TAp73 isoforms were paradoxically overexpressed (18–30 folds) in HNSCC tumor cells in comparison to non-transformed keratinocytes, ΔNp63α was also overexpressed in these tumors and was physically associated with TAp73, thereby inhibiting p73-dependent pro-apoptotic activity (Deyoung 2006; Rocco 2006). *BRCA1*-deficient tumor cells exhibit increased sensitivity to cisplatin, and patients with BRCA1-associated ovarian carcinomas had better outcomes with platinum-based chemotherapy compared with sporadic cases. Ibrahim *et al.* (2010) reported that *BRCA1*-deficient ovarian carcinoma cells exhibited hypermethylation within the P1 promoter for *p73*, which included the binding site for the p73 transcriptional repressor ZEB1, leading to the abrogation of ZEB1-binding and increased expression of transactivating p73 isoforms (TAp73), explaining increased cisplatin sensitivity of *BRCA1*-deficient ovarian carcinomas. Thus, TAp73 might represent a response predictor and potential therapeutic target for enhancing chemosensitivity in ovarian cancer.

Although promoter methylation is the major mechanism of *p73* inactivation in hematopoietic malignancies (Alexandrova & Moll 2012), the situation is different in epithelial tumors - carcinomas. Daskalos *et al.* (2011) studied the DNA methylation status of both P1 and P2 promoters as a means of epigenetic transcriptional control of their corresponding isoforms in 102 primary NSCLCs and reported that the P2 hypomethylation-associated overexpression of *ΔNp73* mRNA is a frequent event, particularly among squamous cell carcinomas. P2 hypomethylation strongly correlated with long interspersed nuclear element-1 element hypomethylation, indicating that ΔNp73 overexpression may be a consequence of global DNA hypomethylation. Guan *et al.* analyzed p73 in prostate cancer and found that ΔNp73 was significantly increased in 20 of 33 prostate carcinomas (Guan & Chen 2005). However, none of the specimens expressed ΔN′p73. The positive expression of ΔNp73 correlated with the Gleason score in prostate cancer. Interestingly, prostate cancer samples with wild-type p53 had significantly higher expression of ΔNp73 than p53 mutant cancers. These data suggested a potential role for ΔNp73 in prostate cancer progression.

Diaz *et al.* (2010) conducted a translational study to evaluate whether 1,25(OH)(2) vitamin D(3) downregulates TP73 variants in colon and breast cancers (Diaz 2010). They reported that ectopic survivin expression led to an increase in all of the TAp73, ΔNp73, ΔEx2p73, and ΔEx2-3p73 transcripts. In these cancers, direct correlations were observed between TP73 variants and survivin levels. Interestingly, 1,25(OH)(2) vitamin D(3) negatively regulated

survivin and TP73 variants in these tumors. Thus positive regulation of TP73 isoforms by survivin may exist, which raised the possibility that the downregulation of TP73 isoforms may be possible with 1,25(OH)(2)D(3) through survivin.

In summary, although somatic point mutations are rarely found in *p73* in human cancers, aberrant splicing that result in ΔTAp73 overexpression are very frequently found. Since these proteins have transdominant activity on all p53 family proteins, it is speculated that this abnormal splicing contributes to human carcinogenesis, esp. in ovarian, breast, lung, and prostate cancers, HNSCCs, and hematological malignancies. Published results indicate that ΔTAp73 overexpression is associated poor clinical outcomes at least in lung cancer and HNSCCs. Of note, it may be possible to correct aberrant expression of p73 isoforms in cancer through the use of 1,25(OH)(2)D(3).

Conclusive remarks

Judging from the very low frequency of mutations for *p63* and *p73* in human cancers, these are not classical tumor suppressor genes, but the possibility remains that these are haplo-insufficient tumor suppressors, just like $p27^{Kip1}$, *PTEN*, or *DMP1* (Quon & Berns, 2001, Berger & Pandolffi 2011; Inoue 2001, 2007; Mallakin 2007; Sugiyama 2008; Taneja 2010; Zhu 2013; Fry 2013). Detailed analyses with specific primers are required to determine whether these are true tumor suppressors. Accumulating pieces of evidence suggest that TA- and ΔN- isoforms play distinct roles in cell cycle progression, apoptosis, and tumor development/prevention. Detection of each isoform by Western blotting or immunohistochemistry with specific antibodies or real-time PCR-mediated quantification of each splicing isoform will be needed to determine the prognostic value of each splicing isoform in cancer. Of note, both ΔNp73 and ΔN′p73 have unique amino acid sequences generated from the exon 3′ that is absent in TAp73. This has made it possible to generate ΔN/ΔN′p73-specific antibodies that can be used in diagnostic immunohistochemistry.

Not many studies have been done to elucidate the mechanisms of overexpression of ΔN isoforms of p63 and p73 in human cancers. Identification of critical splicing factors and characterization of signaling pathways that contribute to this process will be critical to correct the errors for splicing for these genes in human cancers. Finally, specific targeting of ΔN- isoforms with antisense DNA, stabilized RNA, shRNA may have therapeutic values in treating human cancer overexpressing these splicing isoforms with oncogenic activity.

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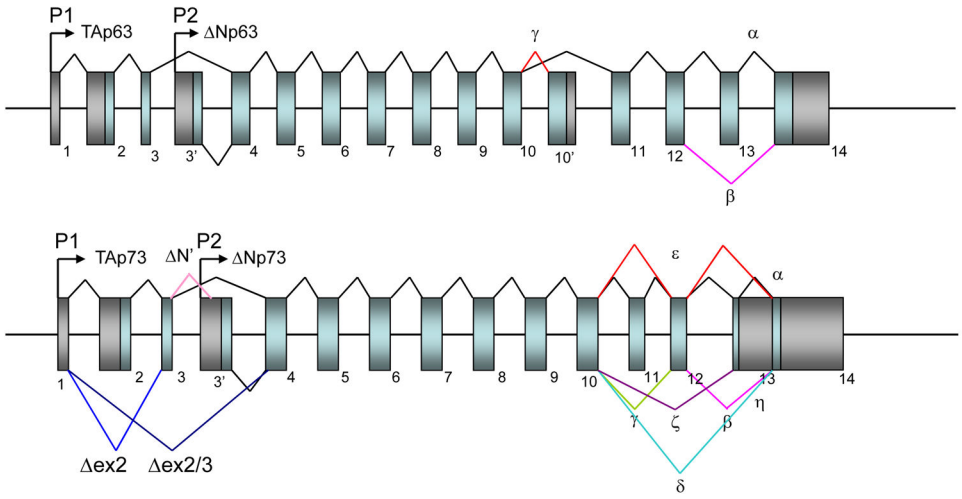

Figure 1. Genomic structure of the p63 and p73 loci

Genomic structures of human *p63* and *p73* loci. Numbered boxes indicate exons, black shading denotes untranslated sequences, and light blue shading denotes coding regions. Distinct transcription start sites (P1 and P2) are indicated by arrows. N-terminal alternative splicing for p63 and p73 are indicated by blue and light pink lines, and C-terminal splicing events for these proteins are indicated by different colored lines.

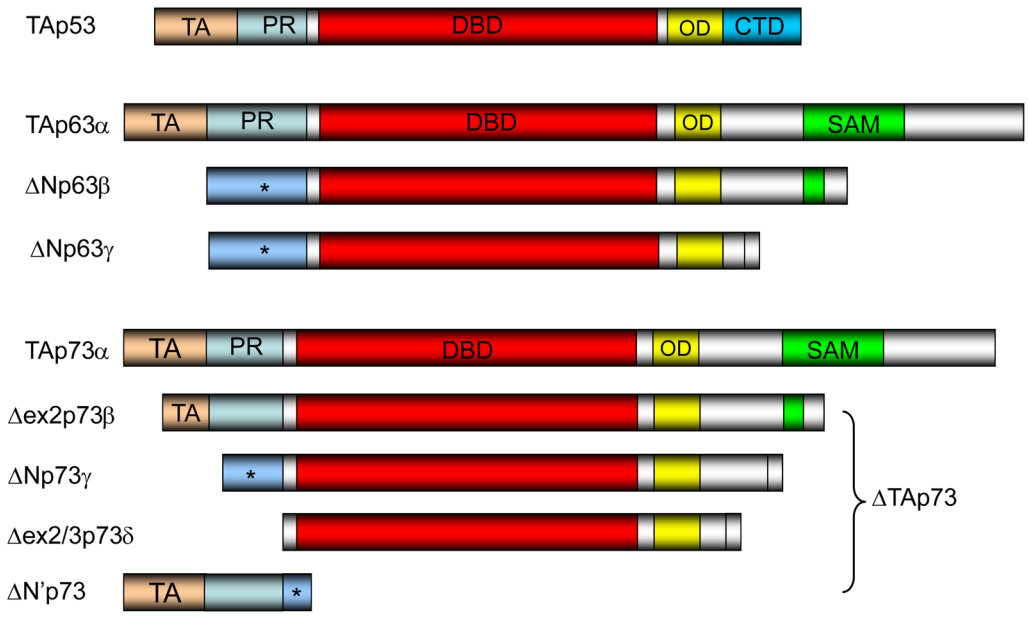

Figure 2. Structure of the p53 family proteins

Protein domains of p53 family members. The transactivation (TA) domains shared by p53, TAp63, and TAp73 isoforms are shown in gold. The proline-rich domain (PR: light blue), DNA-binding domain (DBD: red), oligomerization domain (OD: yellow), carboxyl-terminal regulatory domain (CTD: blue), and sterile alpha motif (SAM: green) are shown in colors. The alpha isoforms of p63 and p73 possess a C-terminal SAM domain followed by a transactivational inhibitory domain (TID: silver). TAp63γ/TAp73γ isoforms most closely look like p53. N-terminally truncated ΔN isoforms for p63 possess the unique N-terminal sequence. p73 has four different isoforms at the N terminus (ΔN, ΔN′, Δex2, Δex2/3) dependent on the usage of two different promoters and alternative splicing including exons 2 and 3. ΔN′p73 encodes a small protein having a unique sequence at the C- terminal end, but lacks the DNA-binding domain. * denotes the unique region encoded by exon 3′ (Murray-Zmijewski 2006).