Minireview

From p63 to p53 across p73

Sabrina Strano[a], Mario Rossi[b], Giulia Fontemaggi[a], Eliana Munarriz[a], Silvia Soddu[a], Ada Sacchi[a], Giovanni Blandino[a,*]

[a] *Molecular Oncogenesis Laboratory, Regina Elena Cancer Institute, Via delle Messi d'Oro 156, 00158 Rome, Italy*
[b] *Dipartimento di Biologia, University of Rome 'Tor Vergata', 00133 Rome, Italy*

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Abstract Most genes are members of a family. It is generally believed that a gene family derives from an ancestral gene by duplication and divergence. The tumor suppressor p53 was a striking exception to this established rule. However, two new p53 homologs, p63 and p73, have recently been described [1–6]. At the sequence level, *p63* and *p73* are more similar to each other than each is to *p53*, suggesting the possibility that the ancestral gene is a gene resembling *p63/p73*, while *p53* is phylogenetically younger [1,2].

The complexity of the family has also been enriched by the alternatively spliced forms of p63 and p73, which give rise to a complex network of proteins involved in the control of cell proliferation, apoptosis and development [1,2,4,7–9].

In this review we will mainly focus on similarities and differences as well as relationships among p63, p73 and p53.

Key words: p63; p53; p73; DNA damage; Transcription; Protein–protein interaction

1. Genomic organization of p53 family members

The genomic organization of the *p53* gene is highly conserved among different species. The human *p53* gene is approximately 20 kb and contains 11 exons [10]. The *p73* and *p63* genes are approximately 65 kb and contain 14 and 15 exons, respectively [1,2]. The p53 family members share a large first intron that is 10.7 kb in *p53* and close to 32 kb in *p73* and *p63* [11]. In *p53*, as well as in *p73*, exon 1 is noncoding and the mRNA derived from this exon might influence translation [12]. Thus, *p53*, *p73* and probably *p63* show a high similarity in the exon/intron organization (Fig. 1A).

The human *p73* promoter has recently been characterized [13]. It has a TATA-like box and displays a low homology to the *p53* promoter [14]. Partial characterization of a large region upstream of the start site of exon 1 has revealed the presence of three E2F sites which may account for the recent finding that p73 expression is triggered, at the transcriptional level, by E2F-1 overexpression [13,15,16] (Fig. 1B). The putative ATG for the p73 variants is located in exon 2. Interestingly, a 1 kb fragment in the large intron upstream of the ATG functions as a silencer suggesting that regulatory elements located in this region may contribute to a tight regulation of p73 expression in different tissues or in response to different stimuli (Fontemaggi and Blandino, unpublished observations) (Fig. 1B). At present, too little is known about the transcriptional regulation of p53 family members to allow firm conclusions. Further characterization of the promoter regions of p73 and p63 will tell us whether the three genes of the family also share common regulatory elements.

2. Structure of p53 family members

Human p53 is translated from a single mRNA with a single open reading frame. It comprises 393 amino acid residues and includes three main functional domains: an N-terminal transactivation domain (TAD), a central DNA binding core domain (DBD) and a C-terminal oligomerization domain (OLD) [17–20]. The integrity of the above mentioned domains is strictly required for the efficient binding of p53 to recognition sites of target genes as well as for transcriptional activation [17]. Unlike *p53*, the *p63* and *p73* genes encode several polypeptides (Fig. 2). Three p63 isoforms, α, β and γ, are translated from RNA molecules that share a common 5′ end and differ at their 3′ end because of alternative splicings [2,4]. Three N-terminal deleted p63 isoforms are generated by a second internal promoter located upstream of exon 3 [2]. Interestingly, these deleted isoforms lose the ability to transactivate target genes and may function as dominant negative of either p63 isoforms or p53 [2]. Two p73 polypeptides were originally identified. The longer one, named p73α, comprises 636 amino acids. The shorter one, named p73β, lacks the C-terminal tail and derives from an alternative splicing of exon 13 [1]. The amino acid sequence of p73β coincides with the 494 amino-terminal residues of p73α with the addition of a short carboxy-terminal tail of five residues. Four additional p73 spliced variants have recently been identified [7–9]. Furthermore, an isoform of mouse p73 truncated in the N-terminus has recently been found and shown to prevent apoptosis in sympathetic neurons after nerve growth factor withdrawal or p53 overexpression [21].

The p63 and p73 proteins display a high homology to p53. The most prominent degree of homology with p53 is found in the DBD (63%) [1]. Furthermore, the critical residues for the proper folding of the entire domain, as well as for the binding to the target DNA sequences, are strictly conserved [1,19]. A lower, but still significant degree of homology to p53 occurs at

*Corresponding author. Fax: (39)-6-49852505.
E-mail: blandino@ifo.it

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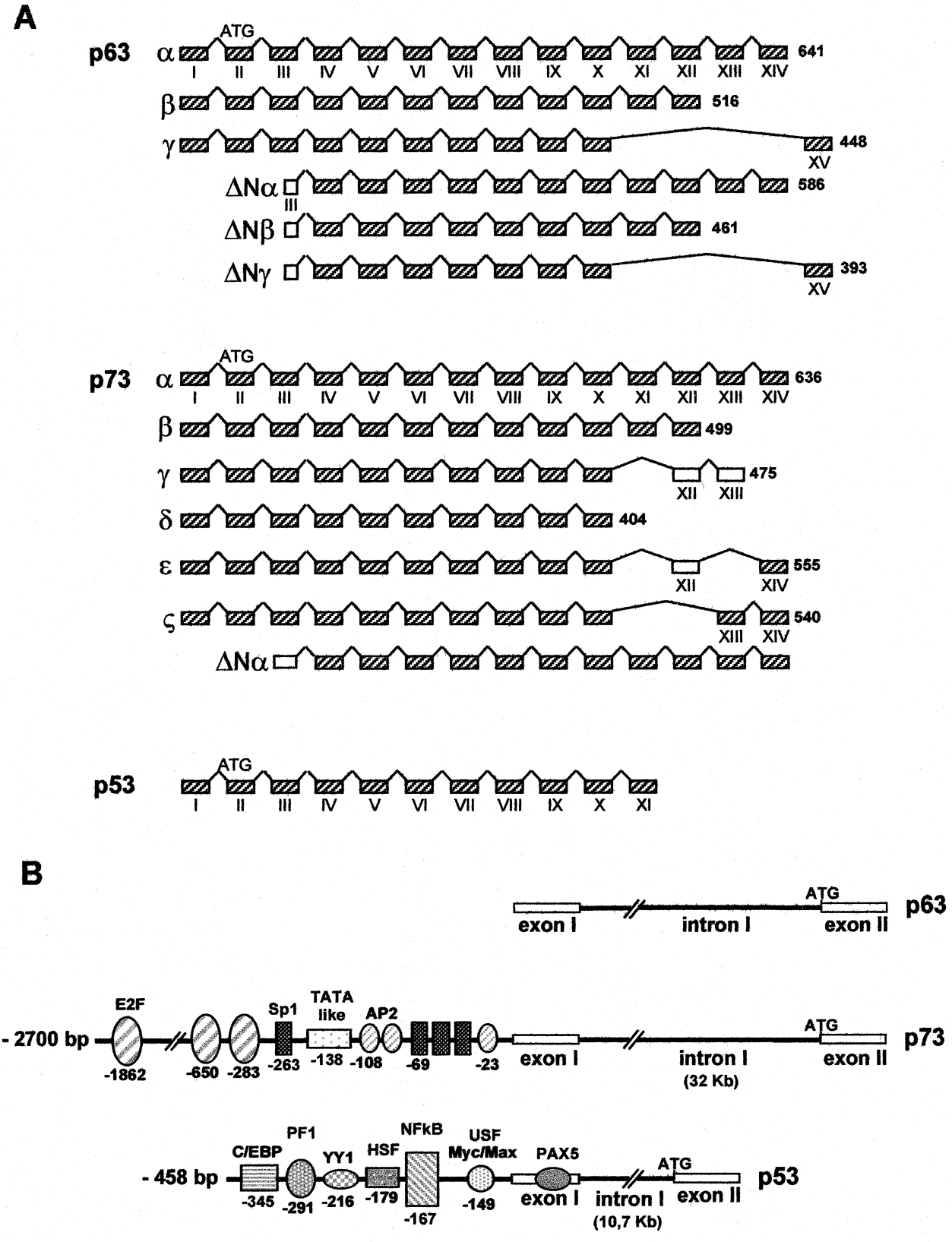

Fig. 1. Genomic organization of p63, p73 and p53. Exon/intron organization of p63, p73 and p53 with their relative isoforms. The sizes of introns and exons are not drawn to scale (A). The promoter region of p63 has not been cloned yet. All genes present a large intron 1 and the ATG is in exon 2. The region upstream of exon 1 is differently organized between p53 and p73 (B). In p73 the positions are relative to the first nucleotide of the published p73 cDNA [1]. In p53 the positions are relative to the major start site for transcription.

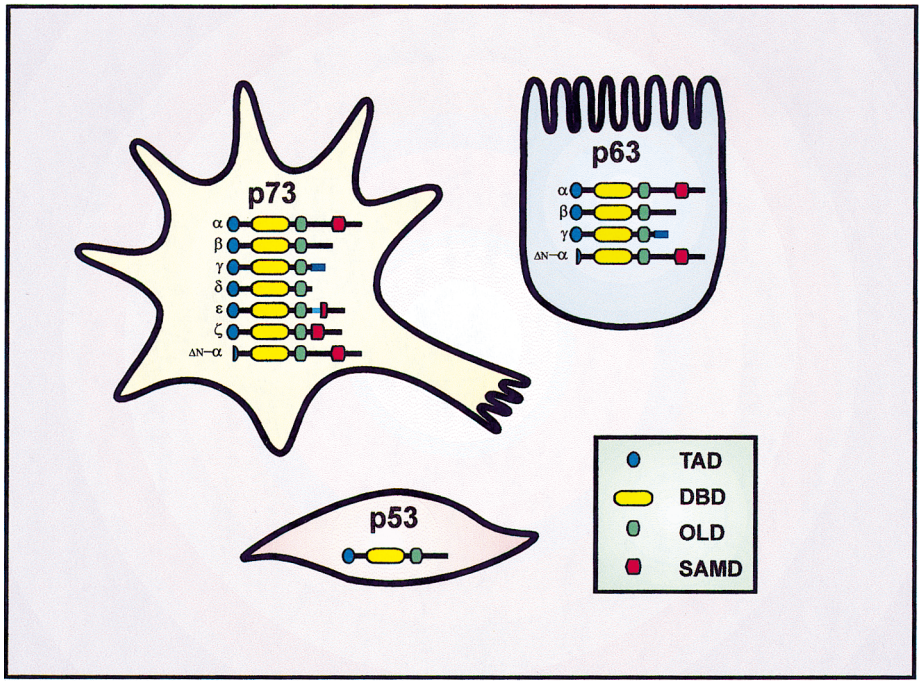

Fig. 2. The protein structure of the different isoforms of the three family members is shown. The major functional implications, according to the knockout mice, are schematically represented by the neuronal cell for p73 and the epithelial cell for p63. The ubiquitous presence and the stress-induced activation of p53 has been exemplified by a generic cell. TAD: transactivation domain; DBD: DNA binding domain; OLD: oligomerization domain; SAM: sterile α motif.

TAD (22–29%) and OLD (42%) of p63 and p73 [22]. The three-dimensional structure of the C-terminal tail of p73 has recently been solved by nuclear magnetic resonance spectroscopy. It consists of a five-helix bundle (487–554 residues) characterized by a marked similarity to the structure of sterile α motif (SAM) domains [23]. These domains are shown to be protein–protein interaction modules present in several cytoplasmic signaling proteins and in transcription factors [24,25]. For instance, SAM-mediated dimerization has been proposed to contribute to Eph receptor activation and self-association of ETS transcription factors. However, by using different approaches, Arrowsmith's group has recently reported that the p73 SAM domain does not homo-oligomerize [23]. Thus, further evidence needs to be collected in order to establish whether the SAM domain modulates p73 and perhaps p63 functions, by either interacting directly with target proteins or modifying the C-terminal tail.

3. Regulation of p53 family members

It has been found that several stress signals strongly and rapidly activate p53. Due to its biological outcomes, including apoptotic death, p53 activity needs to be tightly controlled. Several studies have clearly reported that p53 protein levels increase swiftly in response to DNA damage and to other types of stress, mainly through a significant increase in the protein half-life [26–28]. It has recently been reported that MDM2 is a key player in the regulation of p53 stability. MDM2, the product of a p53-inducible gene, binds to and suppresses p53 activity by promoting its proteolytic degradation [29–32]. p53 induces the expression of MDM2 that, in turn, controls p53 activity and stability, giving rise to an autoregulatory feedback loop. *Mdm2* deficient mice are early lethal but the simultaneous deletion of *p53* rescues this phenotype, indicating that the MDM2-mediated control of p53 activities is crucial for proper development [33,34]. Interference with the binding of MDM2 to p53 by monoclonal antibodies or competitor peptides causes stabilization and accumulation of p53 even in unstressed cells [35]. On the other hand, tumor cells carrying mutant forms of p53 are believed to have elevated levels of p53 protein because of their inability to increase *Mdm2* gene expression, with consequent impairment of MDM2-mediated degradation of p53 [36,37]. Recent studies have shed light on the mechanism by which MDM2 promotes p53 degradation. MDM2 itself shows a specific E3 ubiquitin ligase activity, which is sufficient to covalently attach ubiquitin groups to p53 as well as to itself [38–40]. Nucleo-cytoplasmic shuttling of MDM2 is important in promoting p53 degradation efficiently [41–44]. It has been proposed that MDM2 may be responsible for translocating p53 into the cytoplasm where degradation takes place. However, a nuclear export signal for p53 has recently been identified [45]. These findings indicate that MDM2 and p53 may exit from the nucleus independently. In contrast, no nuclear export signals have been identified in p63 and p73 proteins.

p73 was shown to up-regulate at the transcriptional level the expression of MDM2 that, in turn, reduces p73-dependent transcription in different reporter assays [46]. These findings suggest the existence of a p73/MDM2 regulatory loop similar to the p53/MDM2 loop. However MDM2, by binding to p73α and β, reduces their transcriptional activity but does not induce their degradation [47,48]. Furthermore, in contrast to p53, MDM2 binding promotes stability of p73α and β [49]. Thus, at least with respect to MDM2-mediated degradation, p53 and p73 are clearly divergent.

An additional level of regulation of p53 and p73 is medi-

ated by the covalent addition of SUMO-1 [50–52]. Unlike p73, sumolation of p53 increases its transcriptional activity. If this apparent discrepancy reflects an additional divergence in the regulation of p53 versus p73 needs to be explored by further experiments.

4. Signals from DNA damage activate differently p53, p73 and p63

Over the past few years, many efforts have been focused on understanding the mechanisms underlying p53 stabilization. Many types of DNA damage that cause p53 stabilization have been reported to induce phosphorylation of p53 at specific sites [20,53]. Of particular interest are Ser15, Ser20, Ser37 and Thr18 of human p53, whose phosphorylation reduces the association with MDM2 and consequently protects p53 from degradation [54–58] (Fig. 3). A clear example of the chain of events connecting DNA damage to p53 stabilization is provided by the ionizing radiation-activated ATM kinase that, by phosphorylating p53 at Ser15 reduces its degradation by MDM2 [59,60]. p53 stabilization can also be achieved by phosphorylation of MDM2 that results in reduced association with p53 [61].

Unlike p53, p73 was originally shown not to be induced in response to UV irradiation [1]. Later on, it was reported that cisplatin and ionizing radiation could regulate p73 through protein accumulation or tyrosine phosphorylation, respectively [62–64] (Fig. 3). These post-translational modifications of p73 occur through its physical interaction with the active c-Abl kinase and promote the apoptotic activity of p73 [62–64]. Furthermore, p73 can also be acetylated by p300 upon treatment with cisplatin (Costanzo and Levrero, personal communication). Taken together, these findings indicate that regulation of p73 in response to different types of DNA damage is a complex phenomenon that may be mediated by the recruitment of different upstream proteins that modify p73.

By looking at the overall picture of covalent modifications of p53 family members in response to DNA damage, a striking divergence emerges between p53 and p73. Unlike p53, p73 stabilization seems only to be triggered by a subset of DNA damaging agents. Moreover, the p73 response to stress was found to be mediated by tyrosine phosphorylation while this

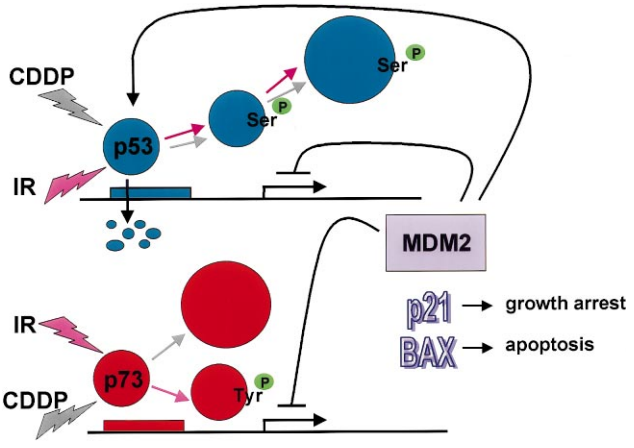

Fig. 3. Schematic representation of the feedback loop between p53/MDM2 and p73/MDM2 in response to ionizing radiation (IR) and cisplatin (CDDP). The biggest circles indicate protein stabilization.

type of modification was never observed in p53 [63,64]. This raises the question on what is the function of p73 tyrosine phosphorylation in response to DNA damage. A simple explanation would suggest that p73 recruits proteins that contain SH2 domains [65]. This would imply that cells exposed to DNA damage recruit a p73-dependent pathway distinct from that activated by p53. For instance, the p73-dependent pathway in response to DNA damage could preferentially be activated in cells that have an inactive p53 protein. Thus, signals generated by DNA damage are integrated by either p53 or p73 to induce specific cellular responses that may also depend on the specific cellular context. Whether the third member of the family, p63, is also involved in mechanisms underlying cell responses to DNA damage needs to be thoroughly investigated.

5. E2F-1-induced apoptosis by activation of p53 or p73

p53 can also be activated in response to oncogenes such as *Ras*, *Myc*, *E1A* and *β-catenin* [66–69]. The molecular mechanism underlying this stabilization has recently been elucidated by the finding that the deregulated overexpression of oncoproteins causes accumulation of p14ARF, a small protein encoded by the *INK4a-ARF* locus [70–72]. p14ARF interacts with MDM2 in a region distinct from the binding domain of p53 and promotes p53 stability through protection from MDM2-mediated degradation [73–76]. Induction of p14ARF is mainly at the transcriptional level and it can be ascribed to E2F transcription factors [77]. Loss of physiological regulation of E2F family is frequently found in human cancers, indicating that deregulated activity of these transcription factors contributes to tumor development [78]. The importance of the integrity of E2F activity in response to oncogenic stress is made apparent by E2F-1-induced apoptosis [79]. Increasing evidence indicates that E2F-1 can induce apoptosis in p53-dependent and -independent ways [80]. Recent work by the Vousden group has shown that exogenous expression of E2F-1 sensitizes p53-null cells to the apoptosis induced by tumor necrosis factor α through the inhibition of anti-apoptotic responses, as reported for activation of NF-kB [81]. An additional mechanism for E2F-1 induction of p53-independent apoptosis has recently been provided by reports that p73 is induced at the transcriptional level by exogenous E2F-1 overexpression in p53-null cells [15]. Induction of p73 by E2F-1 is also triggered by T-cell receptor (TCR)-mediated apoptosis as shown by the reduction of the apoptotic rate upon introduction of a p73 dominant negative [16]. Further support to the functional link between E2F-1 and p73 emerges from the resistance of primary T-cells derived from E2F-1 or p73 deficient mice to undergo TCR-mediated apoptosis [16]. Thus, TCR-activated apoptosis is triggered by a specific pathway in which p73 is not recruited because of p53 absence or inactivation but is the main downstream regulator of apoptosis.

6. p53 family members bind differently to viral oncoproteins

p53 was originally discovered in 1979 as a protein that coprecipitates with the large T antigen of SV40 [82,83] (Table 1). Since this first observation, other viral oncoproteins such as E6 of human papilloma virus and E1B 55 kDa of adenovirus were reported to bind to and inactivate p53 [84,85]. Thus, elimination of p53 activity is considered to be an essential

step for DNA tumor virus transformation [86]. In contrast, none of the above mentioned viral oncoproteins binds or inactivates p73 or p63 [87–89]. However, the possibility exists that they can be bound and inactivated by other viral proteins. Indeed, controversial data have been reported on the ability of E4orf6 of adenovirus to inhibit p73 transcriptional activity [89,90].

Adenovirus E1A promotes p53 stability through p14ARF-mediated sequestration of MDM2 [68,91]. However, E1A can also promote p53 degradation by binding to the transcriptional coactivator protein p300/CBP. The latter associates with the transactivation domain of p53, thereby causing *Mdm2* gene transcription and consequently proteolytic degradation of its product [92–94]. In addition, p300 interacts with both p53 and MDM2 through domains distinct from those involved in transcriptional coactivation [95]. Via these interactions, p300 might allow the assembly of protein complexes required for efficient p53 degradation. Similar to p53, the complex p300/CBP also binds to the N-terminus of p73 promoting its transcriptional activity [89]. Whether acetylation of p73 by p300/CBP, as shown for p53, results in a more efficient recognition of DNA target sequences and in a higher transcriptional activity remains to be established.

7. Phenotypes of $p53^{-/-}$, $p63^{-/-}$ and $p73^{-/-}$ deficient mice

Clues to the physiological roles of p53, p63 and p73 came from the respective knockout mice. The main phenotype of the *p53* deficient mouse is the high incidence of spontaneous tumors, mainly sarcomas and lymphomas [96]. Together with the fact that these mice are frequently viable, these findings strongly indicate that *p53* plays a pivotal role as a tumor suppressor gene [96]. In contrast, *p63* deficient mice are born alive but show striking defects in development. Their skin does not progress from the early stages of development, lacking stratification as well as expression of differentiation markers. The mammary glands, hair follicles and teeth are absent in $p63^{-/-}$ mice [97,98]. In agreement with this phenotype, *p63* was recently found mutated in patients with EEC syndrome whose defects, ectrodactyly, ectodermal dysplasia and facial clefts, closely resemble the phenotype of the $p63^{-/-}$ mice [99].

Table 1
Proteins interacting with the p53 family members

	p63	p73	p53
TAD+proline-rich:			
MDM2	?	+	+
p300/CBP	–	+	+
E1B 55 kDa	–	–	+
TFIID	?	?	+
TFIIH	?	?	+
RP-A	?	?	+
c-AbI	?	+	+
DBD:			
SV40 T Ag	–	–	+
p53BP1	?	?	+
p53BP2	?	?	+
OLD+C-terminal:			
E6 HPV	–	–	+
TBP	?	?	+
XBP	?	?	+
XPD	?	?	+
CSB	?	?	+
E4orf6	–	+	+

p73 deficient mice exhibit severe defects, including hydrocephalus, hippocampal dysgenesis, chronic infections and inflammation, and abnormalities in the pheromone sensory pathway. However, they do not develop any spontaneous tumors [100].

From the phenotypes described, a functional divergence among p53, p63 and p73 clearly emerges. While p53 behaves as a canonical tumor suppressor gene, both p63 and p73 play a major role in ectodermal differentiation and neurogenesis, respectively. However, these findings do not exclude that each member of the p53 family can exert some of the functions ascribed specifically to other members. This would explain why inactivation of p53 interferes with muscle or hematopoietic differentiation in vitro or *Xenopus laevis* development in vivo and, alternatively, why p63 and p73 can recapitulate p53-induced apoptosis as well as growth arrest [1,2,101–108]. Thus, the functions of p53 family members might overlap, at least in specific tissues, as a result of the requirement for concerted and simultaneous activity of p53, p63 and p73 at specific stages of development.

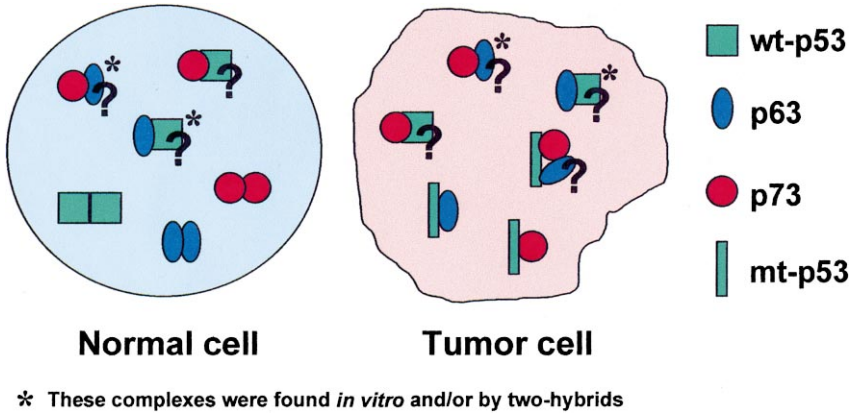

Fig. 4. Homo- and hetero-complexes among the different members of the p53 family and the mutant p53 in tumor and normal cells. The cells were drawn without nuclei since it is not known whether these complexes are exclusively nuclear or they are also present in the cytoplasm. Most of the data available so far on hetero-complexes are from in vitro studies or co-immunoprecipitation in tumor cell lines. Thus, it is possible that the picture is not so divergent between normal and tumor cells. The complexes with a mutant p53 are obvious exceptions to this hypothesis.

8. p53, p73 and p63 in human cancers

The *p53* tumor suppressor gene is the most frequent target for genetic alterations in human cancers [109]. The most prevalent type of p53 mutations consists of missense mutations, often within the highly conserved DBD of the protein [18,20], leading to loss of the wild-type activity. However, at variance with other tumor suppressor genes, cells bearing p53 mutations typically maintain the expression of full-length protein. This may suggest that, at least certain mutant forms of p53 can actively contribute to cancer progression through 'gain of function' oncogenic activity. Such activity might depend on the specific p53 mutation and on the cell context in which the biological outcome of the gain of function is evaluated [110]. We and others have previously reported that conformational mutants such as p53His175, but not DNA contact mutants, can increase cellular resistance to etoposide or contribute to genomic instability by abrogating the mitotic spindle checkpoint and consequently facilitating the generation of aneuploid cells [111–114]. The molecular mechanisms underlying the gain of function activities of mutant p53 remain to be elucidated. We can delineate two mechanisms through which mutant p53 exerts gain of function activities. The first one relies on the assumption that mutant p53 can bind to DNA through the association with DNA binding proteins and activate specific target genes using its functional TAD [115]. In support of this mechanism, it has been reported that human tumor-derived p53, whose TAD was inactivated by site-directed mutagenesis, lost the ability to increase tumorigenicity in vitro and in vivo [116]. In a second scenario, mutant p53 binds to and sequesters proteins whose function is required for anti-tumor functions such as apoptosis or growth inhibition. Interestingly, it has been reported that human tumor-derived p53 mutants can associate with p73α and interfere with its transcriptional activity and ability to induce apoptosis when co-expressed in transient transfection assays [117]. Further studies have demonstrated that the association between mutant p53 and p73 occurs under physiological conditions as indicated by co-immunoprecipitation from various tumor cells [118,119]. Of note, different p73 variants exist in the cells, giving rise to a family of proteins that adds a new level of complexity to the understanding of p73 signaling in cancer cells [1,7,8]. Recent findings indicate that mutant p53 can also be engaged in physical interactions with different isoforms of p73 [119]. The Kaelin group has recently shown that the association between human tumor-derived p53 mutants and p73 is governed by a common polymorphism at codon 72 of p53 that encodes Arg or Pro. Thus, both the type of p53 mutation and the polymorphism at codon 72 influence whether mutant p53 interferes with p73 activity [118]. Heterodimers between mutant p53 and p63 have recently been shown to form in vitro and exist in tumor cells ([118] and Strano and Blandino, unpublished observations), while further evidence needs to be collected to verify whether a triple complex (mt-p53/p63/p73) can assemble in cancer cells. In that case, cancer cells carrying mutant p53 will provide the first and clear example of a context in which p53 family members interact with one another. It will be of interest to verify whether interactions occurring among the p53 family members impact on the chemoresistance of tumor cells (Fig. 4).

While the DBD is the major site of mutations in p53, very rare mutations in p73 and p63 have been found so far despite extensive efforts [120–123]. Interestingly, the DBD of mutant p53 is sufficient for the association with p73 isoforms [119]. The DBDs of mutant p53 proteins have been regarded as 'dead' domains since they cannot bind and activate p53 target genes. However, these DBDs acquire a protein–protein interaction capacity that might contribute to the gain of function activities of mutant p53 by sequestering and inactivating proteins required for anti-tumor functions.

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