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News and Commentary

The functional domains in p53 family proteins exhibit both common and distinct properties

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p53 is a sequence-specific transcription factor that functions to transactivate genes that mediate cell cycle arrest, DNA repair, apoptosis, and other p53-dependent activities. In 1997 and 1998, p73 and p63, respectively, were identified and emerged as p53 homologues (reviewed by Yang *et al.*[1]). The p53 family proteins share significant similarity at the amino-acid level within three domains: the transcriptional activation domain (AD), the sequence-specific DNA-binding domain (DBD), and the tetramerization domain (TD) (Figure 1a). Like p53, both p63 and p73 bind to the canonical p53-responsive element and transactivate p53 target genes (reviewed by Harms *et al.*[2]). Unlike p53, the genes encoding p63 and p73 are rarely mutated in human cancer and knockout mice demonstrate developmental defects rather than a propensity for tumor formation (reviewed by Yang *et al.*[1]). However, recent evidence suggests that p63 and p73 do indeed play a role in tumor suppression since heterozygous p63 and p73 mice are prone to tumor formation.[3] Thus, the p53 family proteins possess both common as well as nonoverlapping functions.

At the 12th International p53 Workshop, we and others presented data identifying the functional domains in the p53 family proteins required for transcriptional activity, cell cycle arrest, and apoptosis (reviewed by Braithwaite *et al.*[4]). As each domain plays an integral role in facilitating the differential functions of these transcription factors, here, we discuss the common and distinct properties of the transcriptional ADs, the DBD, nuclear localization and nuclear export signals (nuclear localization signal (NLS) and nuclear export signals (NES)), the TD, the basic domain (BD) that is present in p53 but not in p63 or p73, and the sterile-α-motif (SAM) domain that is present in some p63 and p73 isoforms but is lacking in p53.

The Transactivation Domain(s) in the p53 Family Proteins

The transcriptional ADs are crucial for the function of the p53 family proteins. Importantly, recent evidence suggests that all three p53 family proteins undergo N-terminal truncation which affects the composition and activity of the AD(s) (Figure 1b and c).

The full-length p53 contains two tandem, independent ADs, whereas ΔNp53 contains only one AD (Figure 1b and c). Full-length p53 is transcribed from the upstream promoter and contains AD1 (residues 1–42) and AD2 (residues 43–92). Previously, AD2 was thought to span residues 43–63. However, we redefine the boundaries of AD2 to include the adjacent proline-rich domain (PRD) (residues 64–92) since both the previously defined AD2 and PRD contribute to the proapoptotic function of p53.[5,6] In addition, data suggest that the transcriptional activity of AD2 in ΔNp63/ΔNp73 requires the PRD (see below for details[7]). ΔNp53, which lacks the first 39 residues, is generated through alternative splicing of intron 2 or alternative translational initiation at codon 40 and contains only AD2 (see this issue review[8]). Both AD1 and AD2 interact with the basal transcriptional machinery as well as histone acetyl transferases and are independently sufficient to activate transcription when fused to a heterologous DBD (GAL4-DBD).[9]

p63 and p73, either as TA or ΔN isoforms, contain only one AD (Figure 1b and c). The TA isoforms are transcribed from the upstream promoter and contain an N-terminal AD1. The p63-AD1 (residues 1–59) and p73-AD1 (residues 1–54) are 22 and 29% identical to AD1 in p53, respectively. The ΔN isoforms are transcribed from a cryptic promoter within intron 3 to yield ΔNp63 and ΔNp73, which lack the first 69 and 62 residues but contain 14 and 13 unique N-terminal residues, respectively. Although ΔNp63/ΔNp73 isoforms lack the AD1 present in the TA isoforms, the ΔN isoforms retain transcriptional activity at least in some settings.[7,10] We have shown that activation of a GAL4-driven reporter by the 13 unique residues of ΔNp73 requires the adjacent PRD, thus the unique residues and the PRD comprise AD2 for the ΔN isoforms.[7] Although the PRD is not required for the activity of AD1 as it is for AD2, the PRD does augment the transcriptional activity of AD1. The AD2 in ΔNp63/ΔNp73 does not share identity with the p53-AD1 or p53-AD2.

The NMR structure of the p53 activation region has recently been solved.[11] Interestingly, the activation region (residues 1–93) was found to be unstructured and natively unfolded.[11] An important characteristic of natively unfolded proteins or domains is their ability to undergo folding upon binding of a target protein.[12] Indeed, many reports demonstrate that the p53 activation region undergoes folding upon binding to interacting proteins such as Mdm2, an E3 ligase and a negative regulator of p53, and hRPA70, a subunit of human replication protein A necessary for DNA replication and repair. Thus, the uncomplicated structure of the ADs facilitates the functional diversity of these domains.

Deletion and mutation of the p53-AD1 indicate that AD1 is dispensable for apoptosis but is required for cell cycle arrest. Specifically, inducible expression of p53 carrying the AD1

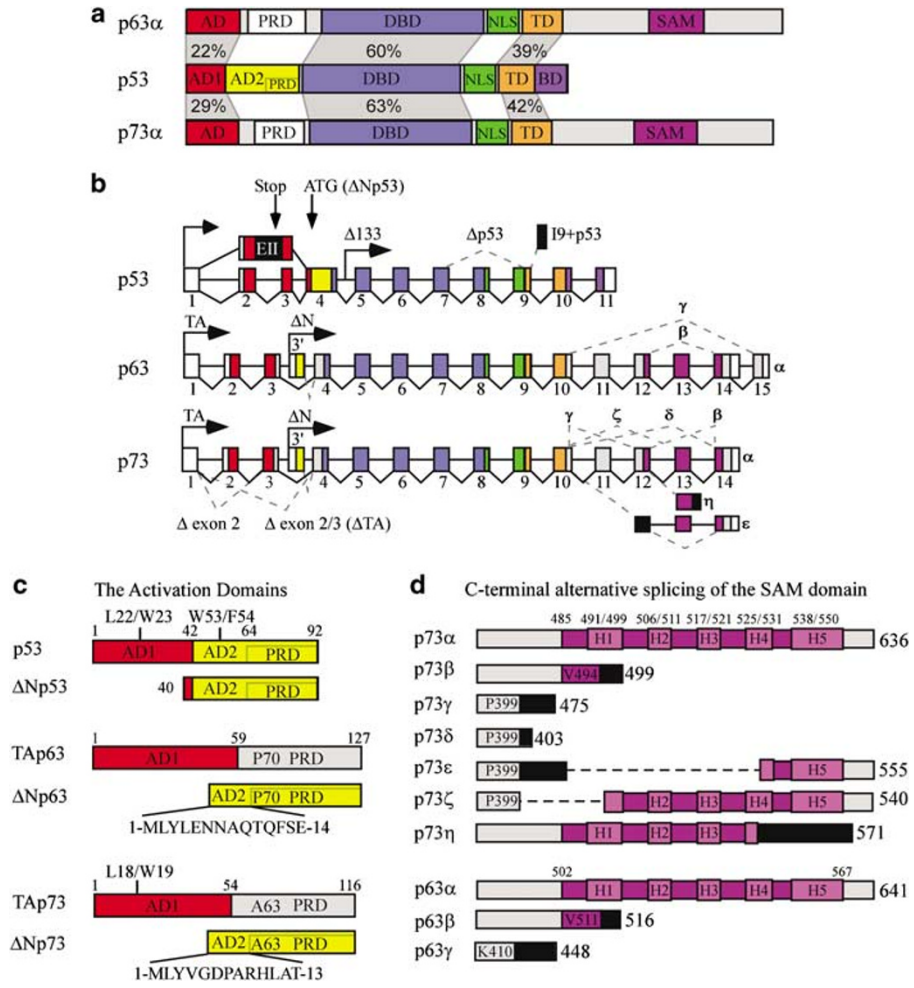

Figure 1 The functional domains in the p53 family proteins. (**a**) AD, activation domain; PRD, proline-rich domain; DBD, DNA-binding domain; NLS, nuclear localization signal; TD, tetramerization domain; BD, basic domain; SAM, sterile-α-motif domain. % denotes percent identity. (**b**) Genomic structure of p53, p63, and p73. Dotted lines denote alternative splicing. Black bars denote an alternative reading frame. (**c**) Composition of the activation domains in p53 family proteins. P53 is transcribed from the upstream promoter, and ΔNp53 is generated by alternative splicing or the use of an internal translational start site. P53 contains AD1 and AD2, whereas ΔNp53 contains only AD2. TAp63/TAp73 isoforms are transcribed from the upstream promoter, and ΔNp63/ΔNp73 isoforms are transcribed from the cryptic promoter within intron 3. TAp63/TAp73 contain AD1. The unique residues and the PRD comprise AD2 in ΔNp63/ΔNp73. Numbers denote amino-acid position. (**d**) C-terminal alternative splicing of p63 and p73 affects the composition of the SAM domain. The five helices in the SAM domain are shown as H1–H5. Black bars denote an alternative reading frame

mutation (L22Q/W23S) results in apoptosis, albeit delayed, but not cell cycle arrest.[13] These data have recently been verified using a p53 knock-in mouse model in which murine p53 contains the AD1 mutation (L25Q/W26S), termed $p53^{QS}$.[14] Embryonic lethality strikingly demonstrates the apoptotic activity of the $p53^{QS}$ mutant. Since both knock-in of $p53^{QS}$ and knockout of Mdm2 result in embryonic lethality, it has been proposed that the lethality associated with the $p53^{QS}$ mouse is due to the lack of negative regulation by Mdm2. Thus, when unregulated by Mdm2, the p53-AD1 point mutant is likely to retain transcriptional activity, at least for a subset of target genes. In line with this, $p53^{QS}$ binds the canonical p53-responsive element and transactivates the *BAX* and *GADD45* genes. Additionally, in response to various stimuli, MEFs expressing the $p53^{QS}$ mutant undergo apoptosis, albeit impaired, but not cell cycle arrest.[14] Can the proapoptotic activities of $p53^{QS}$ be attributed to AD2? To rule out transcriptional-independent mechanisms, the activity of p53 (L22Q/W23S/W53Q/F54S), which contains point mutations that inactivate AD1 and AD2, will need to be addressed under similar conditions. Indeed, when inducibly expressed in H1299 cells, the quadruple mutant is transcriptionally inactive and does not induce cell cycle arrest or apoptosis.[13] Similar to mutation of AD1, deletion of AD1 results in a p53 mutant that induces a transient G1 arrest but is highly proapoptotic.[13] Interestingly, through the use of a transgenic mouse model, overexpression of ΔNp53 has been shown to hyperactivate p53 and induce growth suppression and premature aging through altered IGF signaling.[15] In line with this, we have found that AD1 is inhibitory for while AD2 is required for the induction of the *insulin-like growth factor binding protein 3* (*IGFBP3*) gene by p53.[16] Thus, we speculate that AD1 may mask the function of AD2 to promote premature aging through the alteration of IGF signaling. In summary, a fully functional AD1 is required to induce cell cycle arrest but not apoptosis.

Mutation and deletion of AD2 further confirm that AD2 is essential for p53-dependent apoptosis. p53-AD2 mutants, either through point mutation W53Q/F54S or deletion of residues 43–63 or 64–92, are unable to induce apoptosis but retain a modest ability to induce cell cycle arrest.[6] Similarly, deletion of residues 58–88 of mouse p53 results in an apoptotic defect.[17] When the entire AD2, residues 43–92, is deleted, the p53 mutant is inert.[6] In addition, AD2 is required for the induction of *IGFBP3*, a proapoptotic p53 target gene.[16] Furthermore, phosphorylation of S46 by HIPK2 is necessary for the induction of *p53AIP1*, a proapoptotic gene (reviewed by Scoumanne *et al.*[18]), and the repression of galectin-3, an antiapoptotic gene (reviewed by Braithwaite *et al.*[4]). Interestingly, upon introduction of the phosphorylation mimic (S46F) in p53, clathrin has been shown to interact with p53 and enhance p53-dependent apoptosis (reviewed by Braithwaite *et al.*[4]). In light of these data and the fact that p53-AD1 mutants retain apoptotic activity, AD2 appears to be essential for p53-dependent apoptosis.

For p63 and p73, it is well known that AD1 (present in the TA isoforms) functions as a potent transcriptional AD. Importantly, recent evidence has emerged to demonstrate that the ΔN isoforms also contain a functional AD, called AD2. Since the ΔN isoforms lack the AD which is present within the TA isoforms and *in vitro* studies have shown that ΔNp63/ΔNp73 isoforms can inhibit the function of their TA counterparts, a hypothesis is that the ΔN isoforms act in a dominant-negative fashion over the TA isoforms. In support of this, several reports have demonstrated that the TA and ΔN isoforms are overexpressed in tumor tissue. An alternative hypothesis is that AD2 possesses transcriptional activity. As the signaling mechanisms that positively and negatively regulate the TA and ΔN p63/p73 isoforms are not clearly defined to date, the possibility remains that yet unidentified factors may inhibit the activity of p63 or p73 in tumor cells. Through the use of mutational analysis, we have found that both AD1 and AD2 are competent to induce cell cycle arrest and apoptosis, and thus, we provide evidence to support the latter hypothesis. Although we have found that in general, AD2 is a weaker transcriptional activator than AD1 for many of the known p53 target genes, we have also found that stable cell lines inducibly expressing TA (which contain AD1) or ΔN (which contain AD2) p63/p73 undergo both cell cycle arrest and apoptosis.[7,19] Importantly, deletion of p73-AD1 (residues 1–54) in TAp73β or deletion of the unique portion of p73-AD2 (residues 2–13) in ΔNp73β renders the protein inactive to induce cell cycle arrest and apoptosis.[7] Perhaps AD1 and AD2 have overlapping functions because the domains must function independently since AD1 is only present in the TAp63/TAp73 isoforms and AD2 is only present in the ΔNp63/ΔNp73 isoforms. In addition, we and others have found that some of the ΔNp63 isoforms are able to strongly induce *GADD45*, *FDXR*, *GPX2*, and *Hsp70*.[19–21] In light of data supporting either hypothesis, AD1 present in the TA isoforms is likely to be a more potent transcriptional activator than AD2 in the ΔN isoforms, at least for many known p53 target genes. We speculate that the TA and ΔN homotetramers regulate both common and distinct target genes. As for the TA : ΔN heterotetramers, we speculate that they may have reduced transcriptional activity for p53 target genes but that they may also regulate novel genes or distinct sets of genes. Thus, knowledge of the physiologic TA : ΔN ratio in each tissue type will help us to further understand and define the activities of AD1 and AD2.

As addressed above, the AD composition in all p53 family proteins is regulated by alternative splicing or alternative translational initiation. It will be interesting to learn what governs these activities. In addition to this strict regulation, both p53-AD1 and p53-AD2 are regulated by many common and distinct mechanisms. Here we discuss only a few of the many regulators of AD1 and AD2 since p53 post-translational modifications, p53 partners, and Mdm2 are presented in this issue (see this issue review by Wahl[22]).

For example, AD1 and AD2 are commonly regulated by the associated proteins p300/CBP, Pin1, and Mdm2 and are differentially regulated by the p53 C-terminus itself. The acetyltransferases p300 and CBP are important for p53 target gene transactivation as well as acetylation of p53 itself (reviewed by Jayaraman and Prives[23]). Pin1 positively regulates AD1 and AD2 through isomerization of prolines within AD1 and AD2 (reviewed by Mantovani *et al.*[24] and in this issue by Braithwaite, *et al.*[25]). The resulting prolyl-isomerization and conformational change stabilizes p53, promotes p53 DNA binding, and stimulates transactivation. Pin1 also interacts with p73 to promote the stability and the proapoptotic function of p73 (reviewed by Mantovani *et al.*[24]). Although a putative Pin1-binding site is located within p73-AD1, Pin1 has been shown to interact with S/T-P sites within the C-terminus of p73 (S412/P413, T442/P443 and T482/P483) (reviewed by Mantovani *et al.*[24]). It is not yet known whether Pin1 regulates p63; however, two of the three Pin1-binding sites in p73 are conserved in p63.

Mdm2 is the most critical negative regulator of p53 to date. Mdm2 is well known to interact with residues 17–27 of AD1 to mediate the hyperubiquitination of p53 C-terminal lysine residues as well as to obscure AD1 and interfere with the transactivation of AD1-dependent target genes. Recent evidence demonstrates that residues within AD2 (residues 40–45 and 49–54) and residues within the DBD are also sites of interaction with Mdm2.[26,27] Thus, Mdm2 may negatively regulate both full-length p53 and ΔNp53. Interestingly, Mdm2 has recently been shown to mediate transcriptional repression through the mono-ubiquitylation of histones H2A and H2B (reviewed by Braithwaite *et al.*[4]). Similar to p53, Mdm2 interacts with p63 and within the N-terminus of p73 (reviewed by Melino *et al.*[28]). However, Mdm2 does not mediate the degradation of p63 or p73 as it does for p53. For p73, the interaction with Mdm2 blocks the interaction with p300/CBP and subsequently abrogates p73 transcriptional activity (reviewed by Melino *et al.*[28]). For p63, the interaction with Mdm2 increases the stability and transcriptional activity of p63. Although Mdm2 may not be involved, both p63 and p73 are subject to degradation by the proteosome. Recently, p73 has been demonstrated undergo proteosome-dependent degradation directed by the ubiquitin ligases Itch and UFD2a in ubiquitin-dependent and -independent mechanisms, respectively.[29,30]

The C-terminal BD of p53, residues 364–393, has long been thought of as a regulatory domain. Evidence suggests that this domain is required for efficient transactivation of

many p53 target genes, and may thus be thought to positively affect AD function (reviewed by Braithwaite *et al*.[4] and Liu and Kulesz-Martin[31]). Although NMR data indicate that the BD does not interact with any domain in p53, we have recently demonstrated that the BD negatively regulates the function of AD2.[16] Thus, it appears that AD1 and AD2 may be positively and negatively regulated by the BD, respectively, at least in some circumstances. Taken together, p53-AD1 and AD2 are regulated by both common and distinct mechanisms.

With the expanding list of p53 family isoforms, it has become imperative to systematically evaluate the structure, regulation, and activity of the transcriptional AD(s) within each isoform. It is clear that full length p53 contains two independent tandem ADs (AD1 and AD2) whereas ΔNp53 contains only AD2. In addition, TAp63/p73 isoforms contain AD1 whereas ΔNp63/ΔNp73 isoforms contain AD2. While the AD structure of each isoform may be straightforward, many questions regarding the activity of these domains linger. How do the ADs dictate differential target gene selection? What regulates the mechanism(s) by which the unique AD structures are generated? How do post-translational modifications affect the conformation of the AD structure and the subsequent interaction with associated proteins? How does C-terminal modification, either by alternative splicing or post-translational modification, affect the activity of the AD(s)? How does the recruitment of cofactors to individual target gene promoters affect the activity of the AD(s)? How do the ratios of p53 family isoforms affect the activity of the AD(s)? With further dissection of the p53 family ADs, we are likely to uncover powerful means of regulating the activity of these extraordinary proteins.

DNA-Binding Domain

The DBD is essential for the role of the p53 family proteins as sequence-specific transcription factors. The p53 DBD spans residues 102–292. The p63-DBD (residues 142–321) and p73-DBD (residues 131–310) are 60 and 63% identical to the p53-DBD (Figure 1a). The p53-DBD contains four of the five regions that are conserved among species: conserved region II (residues 117–142), conserved region III (residues 171–181), conserved region IV (residues 234–256), and conserved region V (residues 270–286). These regions are also highly conserved within p63 and p73.

The canonical p53-responsive element contains two decamers or half sites, RRRCWWGYYY, which are separated by a spacer of 0–13 bp, where R = purine, C = cytosine, W = adenine or thymidine, G = guanine, and Y = pyrimidine. Several studies indicate that a monomer binds the pentameric sequence and that a tetramer binds the full consensus site.[32] While many of the known p53 target genes contain the canonical p53-responsive element, other DNA sequences have been identified that are responsive to p53. For example, p53 has been shown to interact with a GC-box (the Sp1 consensus site) within SV40 viral DNA.[33] In addition, p53 regulates the proapoptotic genes, *p53-induced gene 3* (*PIG3*) and *phosphatase of activated cells 1* (*PAC1*), through a pentanucleotide polymorphic microsatellite sequence $(TGYCC)_n$ and a 12-bp palindromic sequence (CCCCACGTGAGG), respectively (reviewed by Harms *et al*.[2]). Although p63 and p73 may bind the canonical p53-responsive element and induce p53 target genes, amino-acid differences among the DBD's may alter the specificity of p63/p73 DNA binding. In line with this, *Aquaporin 3 (AQP3)*, a water and glycerol transporter, is induced by p73, but weakly by p53 (reviewed by Harms *et al*.[2]). Instead of via two half-sites of the p53-responsive element, p73 potentially induces *AQP3* via three half sites in the *AQP3* promoter (reviewed by Harms *et al*.[2]). Similarly, *JAG1* and *JAG2*, ligands of the notch receptor, are induced by p63 and p73, but not by p53 (reviewed by Harms *et al*.[2]). Four half-sites of the p53-responsive element within intron 2 are likely responsible for p63/p73 induction of the *JAG1* gene. Importantly, systematic mutational analysis has revealed that p63 preferentially activates RRRCGTGYYY, which differs from the p53-responsive element within the core CWWG (reviewed by Scoumanne *et al*.[18]). Thus, subtle variation in the p53 family DBD's is likely to impact the binding site selection of the p53 family proteins.

The crystal structure of the p53-DBD bound to DNA has revealed that the conserved regions are crucial for the p53–DNA interaction.[32] The larger part of the DBD forms an antiparallel β-sandwich. This β-sandwich serves as a scaffold that supports the structures important for the interaction with DNA, specifically two large loops and a loop-sheet-helix motif. The two large loops are held together by a zinc atom which is coordinated by three cysteines and one histidine (C176, H179, C238, and C242). C176 and H179 are located in loop 2 which spans conserved region III. C238 and C242 are located in loop 3 which spans conserved region IV. Loop 3 also contains S241 and R248, which contact the DNA phosphate backbone and the minor groove, respectively. The loop-sheet-helix motif spans conserved region V. This motif contains residues that contact the DNA phosphate backbone (R273, A276, R283) as well as the major groove (C277 and R280). One additional residue, K120, is located within conserved region II and is important for the interaction with the major groove and the DNA phosphate backbone. Importantly, all of the residues that are essential for the interaction with DNA are conserved in p63 and p73 except R283, which is a K in p63 and p73 (Table 1).

The importance of sequence-specific DNA binding for p53 to function as a tumor suppressor is underscored by the fact that the majority of tumor-derived mutations are missense mutations. The p53 mutants are classified in one of two categories: contact site (hotspot mutations: R248 and R273) or conformational (hotspot mutations: R175, G245, R249, and R282) (Table 1). Contact site mutants are mutated at a residue crucial for the p53–DNA interaction. Conformational mutants fail to stabilize the β-sandwich core domain and thus lack the appropriate scaffold for the proper interaction with DNA. Interestingly, a recent study investigating the folding and unfolding kinetics of the p53-DBD found that wild-type and conformational mutant (G245S, R249S, and R282Q) p53-DBD's folded with similar kinetics; however, the mutants displayed an accelerated unfolding rate.[34]

Although the p63 and p73 DBD's do not appear to be mutated in human cancer, missense mutation of the p63-DBD is associated with several autosomal dominantly inherited syndromes. While these syndromes display common features

Table 1 DNA binding domain: residues involved in DNA binding and hotspot mutation sites

	p53	p63	p73
DNA contact sites			
Major groove	K120	K149	K138
	C277	C308	C297
	R280	R311	R300
Minor groove	R248[a]	R279[a]	R268
Phosphate backbone	K120	K149	K138
	S241	S272	S261
	R273[a]	R304[a]	R293
	A276	A307	A296
	R283	K314	K303
Hotspot conformational mutation sites	R175	R204	
	G245		
	R249	R280	
	R282		

[a]Hotspot contact site mutation sites

such as limb malformations, facial clefting, and ectodermal dysplasia, it is interesting that the p63 mutations are distinctly associated with each syndrome. For example, acro-dermato-ungual-lacrimal-tooth (ADULT) syndrome is associated with mutation of R298; split hand/foot malformation (SHFM) Syndrome is associated with mutation of K193, K194, and R280; and EEC (ectrodactyly, ectodermal dysplasia, facial clefting) Syndrome is associated with multiple mutations with the most frequent being of R204, R227, R279, R280, and R304 (reviewed by Brunner *et al*.[35]). The most frequent mutations in p63 correspond to p53 hotspot mutations: p63-R204 and p63-R280 are analogous to the p53 conformational mutants R175 and R249, respectively, and p63-R279 and p63-R304 are analogous to the p53 contact site mutants R248 and R273, respectively (Table 1).

The activity of p53 can be affected by modification of the DBD. Interestingly, recent evidence suggests that alternative splicing of the p53-DBD generates a novel p53 isoform, termed Δp53 (Figure 1b).[36] Δp53(Δ257–322) lacks 66 residues that include conserved region V and the NLS. Although five of the eight DNA contact sites in the DBD are deleted, chromatin immunoprecipitation (ChIP) analysis showed that Δp53 retains the ability to bind the promoters of p53-responsive genes that mediate cell cycle arrest, but not apoptosis.[36] In irradiated cells, it was shown that the *CDKN1A* promoter was immunoprecipitated by PAb DO12 (which recognizes full-length p53) only in G1 and G2 phases, but by PAb DO1 (which recognizes both full length and Δp53) in G1, S, and G2 phases.[36] These data imply that p53 regulates *CDKN1A* during G1 and G2, while Δp53 regulates *CDKN1A* during S phase.[36] Thus, it appears that alternative splicing of the DBD can promote p53-dependent cell cycle arrest over apoptosis during the intra-S phase checkpoint.

The p53-DBD regulates its own activity in DNA binding. It has been shown by several groups that a p53-DBD fragment, which lacks the TD, cooperatively binds DNA as well as undergoes conformational changes in the β-scaffold upon DNA binding.[37] Through multiple techniques, solvent-exposed residues were identified within the H1 helix in loop 2 (which spans the conserved region III). These residues are proposed to form the interface between two DBD's. Unlike the p53-DBD, the p63-DBD does not cooperatively bind DNA.[38] When artificially dimerized with GST, the p63-DBD is capable to bind the p53 consensus site.[38] Although p63 and p73 demonstrate a high % identity to p53 within conserved region III, the primary sequences within the H1 helix vary. It has been proposed that these differences inhibit the dimerization of the p63-DBDs and, thus, cooperative p63 DNA binding.[38] It is not known whether p73 demonstrates cooperative DNA binding.

Proteins that interact with the p53-DBD and positively affect p53 activity include 53BP1, apoptotic-stimulating protein of p53 1 (ASPP1) and ASPP2. The crystal structures indicate that both 53BP1 and ASPP2 interact with loops 2 and 3 (which span conserved regions III and IV) in the p53-DBD. 53BP1 stimulates p53 transcriptional activity, at least for the *CDKN1A* gene.[39] ASPP1 and 2 have been shown to stimulate p53-dependent apoptosis and augment the activation of pro-apoptotic promoters such as *BAX* and *PIG3* (reviewed by Scoumanne *et al*.[18]). Importantly, ASPP1 and 2 also interact with p63 and p73 and stimulate the proapoptotic functions of these proteins as well (reviewed by Scoumanne *et al*.[18]). It was reported at the 12th International p53 Workshop that the ras-signaling pathway is upstream of ASPP-stimulated p53-dependent apoptosis (reviewed by Braithwaite *et al*. and in the issue by Braithwaite *et al*.[25]).

The p53-DBD also interacts with proteins that negatively regulate p53 such as Mdm2 and a viral oncoprotein of SV40, the large T antigen. While Mdm2 is known to interact with the N-terminus of p53, it has also been shown that Mdm2 interacts with residues in the DBD that connect conserved regions IV and V.[26] In line with this observation, it was recently shown that the binding of Mdm2 to full-length p53 is 10-fold stronger than binding to the N-terminal domain alone.[11] The Mdm2 interaction with the DBD results in hyperubiquitination of p53 that is dependent upon Mdm2 interaction with the N-terminal region.[26] The large T antigen promotes tumorigenesis of SV40 by binding to and inactivating p53.

Thus, it appears that the p53 family proteins have a highly conserved DBD and are commonly regulated by the ASPP family of proteins. However, the proteins also possess unique functions and regulate distinct downstream target genes. Subtle differences in the DBD's, such as the one within the H1 helix, are likely to contribute to the distinct functions of the transcription factors.

Nuclear Targeting Regions

p53 is known to rapidly shuttle between the nucleus and the cytoplasm. Since tetrameric p53 is too large to passively diffuse across the nuclear pore, its nucleo-cytoplasmic shuttling is facilitated by nuclear import and export signals. p53 has a bipartite NLS (residues 305–322) and N- and C-terminal NES (residues 11–27 and 340–351, respectively). Interestingly, the C-terminal NES is embedded within the TD (residues 326–356, see next section for details). The basic residues at the N- and C-termini of the NLS ($K^{305}R^{306}$/$K^{319}K^{320}K^{321}$) are necessary and sufficient for the complete nuclear localization of a cytoplasmic reporter protein.[40] In

addition, the p53-NES is sufficient for nuclear export of FITC-labeled BSA (reviewed by Scoumanne *et al*.[18]). A bipartite NLS (residues 327–348) and NES (367–378) have also been identified in p73, which correspond to those in p53 (reviewed by Scoumanne *et al*.[18]). The p73-NLS and -NES are sufficient for nuclear import and export of cytoplasmic reporter proteins, respectively (reviewed by Scoumanne *et al*.[18]). Sequence alignment shows that the basic residues necessary for the p73-NLS and the p73-NES are conserved in p63. Since p53, p63, and p73 contain both a NLS and NES, it will be interesting to learn how nucleo-cytoplasmic shuttling affects the activity of the transcription factors. For example, to date, mechanisms that regulate p53 nuclear export and degradation by Mdm2 remain unclear (reviewed by Michael and Oren[41]). In addition, with the recently characterized proapoptotic function of p53 at the mitochondria, a new light is shed on the functional importance of p53 nuclear export.

Interestingly, the p53-NLS and N-terminal p53-NES are subject to post-translational modifications. In the p53-NLS, S313, and S314 are phosphorylated by Chk1 and Chk2, S315 is phosphorylated by CyclinA/cdk2, and K320 is acetylated by P/CAF (reviewed by Scoumanne *et al*.[18] and Ou *et al*.[42]). Phosphorylation of S315 and acetylation of K320 have been shown to stimulate p53 sequence-specific DNA binding. It is important to note that S313, S314, S315, and K320 are not conserved in either p63 or p73. Thus, these modifications may play a role in the distinct activities of p53 compared to p63 and p73. In addition, it is known that cytomegalovirus infection results in the cytoplasmic sequestration of p53 that is dependent upon the p53-NLS.[43] Thus, it appears that p53 activity may be positively affected by post-translational modifications within the NLS, but that this region may also be targeted by viral proteins to inhibit p53 activity. In the N-terminal p53-NES, S15, and S20 are phosphorylated in response to DNA damage. Aspartic acid substitution of S15 and S20 resulted in decreased nuclear export, suggesting that phosphorylation of the N-terminal p53-NES could function to inhibit this domain.[44]

The TD in p53 Family Proteins

For high-affinity DNA binding and transcriptional activation, p53 must be in the tetrameric form. Tetramerization of p53 is mediated through the TD (residues 326–356) (reviewed by Chene[45]). The p63-TD (residues 360–390) and p73-TD (residues 353–383) are 39 and 42% identical to that in p53, respectively (Figure 1a). Crystallographic and NMR studies have determined that p53 is a dimer of dimers (reviewed by Chene[45]). The secondary structure of the p53-TD is a β-strand linked to an α-helix by Gly334. Although the p63-TD and p73-TD structures have not yet been solved, residues flanking the conserved Gly (Gly368 in p63 and Gly361 in p73) predict the secondary structure of a β-strand linked to an α-helix. In addition, these domains tetramerize *in vitro* (reviewed by Chene[45]). p53 monomers dimerize through anti-parallel β-sheet and antiparallel helix interactions. Dimers tetramerize through parallel helix–helix interactions. The tetramer is stabilized through hydrophobic forces generated by the helix–helix interaction. Mutation of the hydrophobic core (Leu344Ala) is sufficient to dissociate the tetramer into dimers (reviewed by Chene[45]). The Leu is conserved in p73 (Leu371) but is an Ile in p63 (Ile378). While the tetramer is generally thought to be a dimer of dimers, it is interesting that a recent study using molecular dynamic simulations found that tetramerization is likely to occur as a single event with the folding and association of the dimers occurring simultaneously.[46]

Although the TD is not a mutational hotspot, mutation of this domain has been found to be causative of Li-Fraumeni syndrome in some families.[47] The L344P mutation lies within the α-helix that is important for the dimer–dimer interaction and results in monomeric p53. The R337C mutation also lies within the α-helix. The R337C p53 mutant is not fully tetrameric and also lacks stability.[47]

Thus, it appears that tetramerization is essential for p53 to function as a tumor suppressor. In line with this, several studies support the requirement of tetramerization for many of the well-characterized properties of p53. For example, DNA damage-induced signaling to p53 mediated by phosphorylation of S15, S20, and S33 requires the TD but not other domains (reviewed by Michael and Oren[41]). Interaction with Mdm2 is also impaired when p53 is not in the tetrameric form. In addition, p53 lacks DNA-binding activity *in vitro* upon disruption of the hydrophobic core that stabilizes the dimer–dimer interaction (reviewed by Chene[45]).

With the knowledge that multiple p53 family isoforms exist in both wild-type and mutant forms (reviewed in this issue by Bourdon[8]), it becomes important to evaluate the role of heterotetramerization. The first consideration is the consequence of heterotetramerization between p53 and mutant p53 or other p53 isoforms. The heterotetramerization of wild-type and mutant p53 is likely causative of the dominant-negative activities of mutant p53. It will be interesting to learn how heterotetramerization of full-length p53 and ΔNp53 (which lacks the 39 N-terminal residues) affects p53 activity. It is already known that the premature aging phenotype of the ΔNp53 mouse requires the presence of full-length p53.[15] Thus, is the transcriptional activity of the heterotetramer different from that of the homotetramer? Likewise, heterotetramerization of p63 (or p73) isoforms (the ΔN with the TA isoforms, complicated by the variations at the C-terminus) certainly affects the activities of p63 (or p73).

Another consideration is whether the TD's of p53, p63, and p73 may heterotetramerize. Although the overall secondary structure of the TD in p53, p63, and p73 is conserved, differences in the primary sequence at residues that stabilize the hydrophobic core make heterotetramerization among p53 family proteins unlikely. In support of this, human p53 does not efficiently heterotetramerize with Xenopus p53, which are 52% identical in the TD (reviewed by Chene[45]). However, a human p53 chimeric, which contains the Xenopus TD, is able to heterotetramerize with Xenopus p53. Thus, the specific residues that stabilize the hydrophobic core are crucial for tetramer formation (reviewed by Chene[45]). In addition, *in vitro* studies have determined that the p53-TD does not interact with the TD's in p63 or p73 (reviewed by Chene[45]). However, a very weak association was detected between the TD's of p63 and p73 *in vitro* (reviewed by Chene[45]). The lack of heterotetramerization of p53 with p63 or

p73 has great functional importance. For example, we and others have found that mutant p53 does not have a dominant-negative effect on the ability of p73 to activate transcription or to induce apoptosis (reviewed by Chene[45] and Willis *et al*.[48]). Similarly, a p73 DNA binding mutant was not able to inhibit the transcriptional activity of p53, but it was able to block the activity of p63 (reviewed by Chene[45]).

Thus, the TD's are highly conserved and are required for the proper function of the p53 family proteins. Interestingly, the TD's have distinct properties that prevent the heterotetramerization of the p53 family proteins. This is significant for the function of these proteins since p53, p63, and p73 are likely to be expressed simultaneously, at least in some tissues.

Basic Domain

p53, but not p63 or p73, contains a C-terminal BD which spans the last 30 residues (residues 364–393). The BD has been subjected to extensive analyses and all evidence suggests that it is a regulatory domain. A recent NMR study indicates that the BD is similar to ADs 1 and 2 in that it does not form a regular secondary structure.[49] The unrestricted structure facilitates diversity in the regulation of and by the BD. Since the BD is located at the extreme C-terminus, this domain could fold upon binding a target protein or serve as a flexible linker. The BD has been found to both positively and negatively regulate p53 activities. This is likely due to the fact that nearly every residue within the BD is subjected to at least one post-translational modification such as phosphorylation, methylation, acetylation, ubiquitination, neddylation, or sumoylation. For further discussion about the BD, see reviews in this issue.[31,50]

Sterile-α-Motif Domain

Instead of the C-terminal BD that is present in p53, the α isoforms of p63 and p73 contain a C-terminal SAM domain. The p63-SAM domain spans residues 502–567 and the p73-SAM spans residues 485–550 (Figure 1d). Crystallographic and NMR studies demonstrate that the SAM domain in p73 is a globular five-helix bundle (reviewed by Arrowsmith[51]). The antiparallel interaction of helices 1 and 5 create a hydrophobic surface for the association of helices 2, 3, and 4 (reviewed by Arrowsmith[51]). The structure of p73-SAM is similar to the SAM domain in other proteins such as in the Eph family of receptor tyrosine kinases and the ETS family of transcription factors (reviewed by Yang *et al*.[1]). Interestingly, a SAM domain is found in the C-terminus of the Squid p53-like gene, whereas the p53 of higher organisms does not contain a SAM domain. Squid possess a single p53-like gene which is more similar to p63 and p73 than to p53. Because of these, it has been proposed that p63 is the ancestral gene and that p73 and p53 are the result of gene duplications, with the latter losing the SAM domain (reviewed by Yang *et al*.[1]).

In general, SAM domains are thought to mediate protein–protein interactions. SAM domains mediate interactions through homo- or heterodimerization or through direct interaction. Although p63 and p73 contain functional TDs and do not require their SAM domains for oligomerization, it is surprising that several techniques have clearly demonstrated that the p63-SAM and the p73-SAM do not homo- or heterodimerize (reviewed by Arrowsmith[51]).

Since the p53 gene in higher organisms lacks the SAM domain, it is likely that the SAM domain is important for the differential activities of p63 and p73. Indeed, the SAM domain is important for p63's role in development since naturally occurring mutations in the SAM domain are associated with several dominantly inherited syndromes such as ankyloblepharon, ectodermal dysplasia, and clefting (AEC) syndrome or Hays–Wells syndrome, limb-mammary syndrome (LMS), and EEC. Although the majority of mutations in p63 are within the DBD and are causative of EEC and to a lesser extent ADULT and SHFM syndromes, the majority of mutations within the SAM domain are causative of AEC syndrome (reviewed by Brunner *et al*.[35]). The missense mutations are G518L, F526C, I534G, I537T, Y540Q, and S541I. The former three residues are conserved in the p73-SAM while the latter are not. EEC is associated with only one frameshift mutation within the SAM while LMS is associated with two frameshifs. AEC is characterized by limb abnormalities, ectodermal dysplasias such as alopecia, scalp infections, dystrophic nails, hypodontia, ankyloblepharon, cleft lip/cleft palate, and malformation of the facial skeleton, urogenital system, and eyes. LMS is characterized by hand/foot abnormalities and hypoplasia/aplasia of the mammary gland and nipple. EEC is characterized by ectrodactyly, ectodermal dysplasia, and facial clefting. Thus, the SAM domain is crucial for the role of p63 in development. To date, no mutation of the p73-SAM has been reported to be associated with or causative of a developmental syndrome.

We and others have made the observation that the C-terminally truncated p73β isoform is more active to induce apoptosis as well as p53 target genes than the full-length p73α counterpart. p73β is C-terminally truncated through alternative splicing of exon 13, which results in replacement of the 142 C-terminal residues (corresponding to the 2nd half of helix 1 within the SAM domain to the end) with five different residues (Figure 1d). Systematic mutation of the p73-SAM domain has revealed that it plays a role in negatively regulating the transcriptional activity of p73. The reduced activity of p73α compared to p73β is not due to decreased p73 DNA binding, since deletion of the SAM domain in p73α does not affect p73 DNA binding as determined by ChIP.[52] Through the use of chimeric proteins, we found that the p73-SAM domain negatively regulates the transcriptional activity of p73α.[52] Specifically, the p73-AD is sufficient to activate transcription when fused to a heterologous DBD (GAL4-DBD). However, addition of the SAM domain inhibits the activity of the p73-AD.[52] Similarly, the p73-SAM domain inhibits the transcriptional activity of the p53-AD1 as well as the GAL4-AD.[52] Interestingly, both the SAM domain and the extreme C-terminus in p73α are independently sufficient to inhibit the transcriptional activity of p73α since the presence of either domain is inhibitory and inactivation of both domains is required to relieve the inhibition.[52] When both domains are deleted, the mutant p73α has transcriptional activity and apoptotic ability similar to p73β.[52] However, upon deletion of the extreme C-terminus, an intact SAM domain is required for the inhibitory activity since deletion of helix 5 alone partially

releases the inhibition and combined deletion of H4-H5, H3-H5, or H1-H5 results in a stepwise release of the inhibition.[52] Importantly, the SAM domain, along with the extreme C-terminus, inhibits the transcriptional activity of p73α by blocking the interaction of p73α with the transcriptional coactivators p300 and CBP.[52] Thus, the SAM domain, along with the extreme C-terminus, negatively regulates the transcriptional activity of p73. In line with these, the p63-SAM domain and the p63 extreme C-terminus have also been identified as inhibitory domains (reviewed by Scoumanne *et al.*[18]).

Since the SAM domain is a negative regulatory domain for full length p63 and p73 isoforms and is crucial for the function of p63 in development, it is interesting that alternative splicing regulates the presence of this domain in p63 and p73. Alternative splicing of the C-terminus results in three p63 isoforms (α-γ) and at least seven p73 isoforms (α-η) (Figure 1b and d) (reviewed by Harms *et al.*[2]). Only the full-length α isoforms of p63 and p73 contain an intact SAM domain (Figure 1d). The C-terminally truncated isoforms p63β, p63γ, p73β, p73γ, and p73δ totally lack the SAM domain, whereas p73ζ lacks the first half of helix 1, p73ε lacks helices 1–3 and the first-half of helix 4, and p73η lacks the secnd-half of helix 4 as well as helix 5 (Figure 1d). The different isoforms display a range of transcriptional activities, at least for well characterized p53 target genes, with the β isoforms being the strongest transcriptional activators.

Thus, the SAM domain is dispensable for tumor suppression but required for the distinct activities of p63 and p73. While this domain is well known to be regulated by alternative splicing, it has recently been demonstrated that the p73-SAM binds to both anionic and zwitterionic lipids.[53] It will be interesting to learn how the p73–lipid interaction in combination with alternative splicing regulates the localization and function of p73. Future studies need to further characterize the physiological significance of the p73-SAM–lipid interaction as well as identify proteins and post-translational modifications that affect the activity of the SAM domain. In addition, it would be exciting to learn what regulates the alternative splicing of this domain. Could we manipulate a tumor cell to express the highly pro-apoptotic p63β or p73β isoform?

Concluding Remarks

It is clear that the p53 family proteins are comprised of several functional domains and that the ADs, the DBDs, and the TDs are highly conserved. While all p53 family proteins are now thought to play a role in tumor suppression, it is well known that p63 and p73 play distinct roles in development. Thus, although recent studies have revealed several common and some unique features of these domains, it remains a challenge to discover the distinct regulation and activities of each domain. For example, what regulates alternative splicing of the ADs in p53, p63 and p73 or of the SAM domain in p63 and p73? How does the homo- and heterotetramerization of the alternatively spliced products affect the activity of the transcription factors?

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