Cell Death and Differentiation (2010) 17, 901–911

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Review

p53-family proteins and their regulators: hubs and spokes in tumor suppression

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The tumor suppressor p53 is a central hub in a molecular network controlling cell proliferation and death in response to potentially oncogenic conditions, and a wide array of covalent modifications and protein interactions modulate the nuclear and cytoplasmic activities of p53. The p53 relatives, p73 and p63, are entangled in the same regulatory network, being subject at least in part to the same modifications and interactions that convey signals on p53, and actively contributing to the resulting cellular output. The emerging picture is that of an interconnected pathway, in which all p53-family proteins are involved in the response to oncogenic stress and physiological inputs. Therefore, common and specific interactors of p53-family proteins can have a wide effect on function and dysfunction of this pathway. Many years of research have uncovered an *impressive* number of p53-interacting proteins, but much less is known about protein interactions of p63 and p73. Yet, many interactors may be shared by multiple p53-family proteins, with similar or different effects. In this study we review shared interactors of p53-family proteins with the aim to encourage research into this field; this knowledge promises to unveil regulatory elements that could be targeted by a new generation of molecules, and allow more efficient use of currently available drugs for cancer treatment.
Cell Death and Differentiation (2010) **17,** 901–911; doi:10.1038/cdd.2010.35; published online 9 April 2010

After its first description as a nuclear protein engaged by the oncogenic SV40 large T antigen, and the realization, years later, that p53 is a powerful tumor suppressor, the scientific community has invested a formidable effort on understanding its function and regulation.[1] p53 is a transcription factor whose activity is promoted by a wide range of stress signals potentially affecting genome integrity and proper cell proliferation; once activated, p53 is capable of coordinating a complex cellular response that leads to cell-cycle arrest, DNA repair, senescence, or programmed cell death. For this crucial function, p53 was suitably dubbed the 'guardian of the genome'.[1] More than 15 years after discovery of p53, two p53-related genes were identified: p63 and p73.[2–4] Interestingly, p63 and p73 are structurally similar and functionally related to p53, and hence the entire p53 family may be regarded as a unique signaling network controlling cell proliferation, differentiation, and death.

In this study we present an overview of protein interactors of all p53 family members, with emphasis on their possible role in mediating integrated functions of the p53 pathway. For an update on the complexity of p53 activities and regulation, the reader is referred to specific recent reviews (e.g., Kruse and Gu[5], Vousden and Prives[6], Green and Kroemer,[7] and Menendez *et al.*[8]).

All p53-family Proteins are Involved in Tumor Suppression

All three p53-family proteins have a very similar domain organization, are expressed in a similar set of alternative isoforms, and are subject to similar post-translational modifications (PTMs; summarized in Figure 1); however, mouse models revealed important differences in their biological role, showing that p53-family paralogs have acquired a high degree of functional specificity since their duplication and divergence during evolution.[9,10] p53-null mice are viable and largely normal in embryonic development, but die of cancer at young age, highlighting the crucial role of p53 in preventing formation of spontaneous tumors.[11] Mild developmental and fertility defects can be detected with careful analysis.[12,13] On the contrary, p73-null mice are born viable but have nervous system abnormalities, hydrocephalus, and immunological problems with chronic inflammation. p73-null mice also show reproductive and behavioral defects, and generally die within the first 2 months.[4] p63-null mice are born alive, but die immediately after birth. They show a severe phenotype, lacking limbs and a wide range of epithelial structures including skin, prostate, breast, and urothelia,[2,3] indicating that p63 is required to maintain the pool of proliferating stem cells during development of epithelia.[14,15]

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Keywords: p53 family; protein–protein interactions; tumor suppressor genes
Abbreviations: PTM, post-translational modification; MDM2, mouse double minute-2; YAP-1, Yes-associated protein 1; TAF, transcription-associated factor; JMY, junction-mediating and regulatory protein; PML, promyelocytic leukemia protein; AFP, α-fetoprotein; TRAIL, TNF-related apoptosis-inducing ligand; mut-p53, mutation of p53; PML-NB, PML nuclear body; R-Smad, receptor Smad
Received 12.1.10; revised 22.2.10; accepted 01.3.10; Edited by G Melino; published online 09.4.10

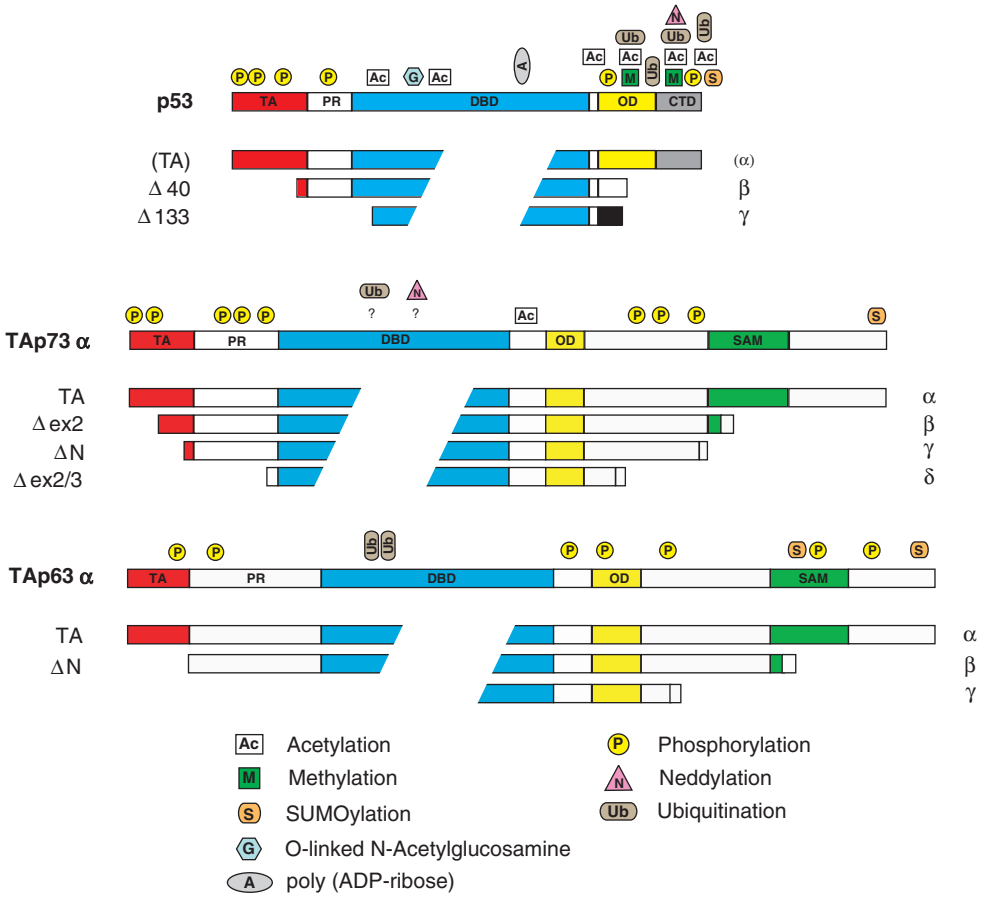

Figure 1 Structure of p53-family proteins and their principal isoforms, together with some regulatory post-translational modifications. p53 family proteins have a similar structure, comprising an N-terminal transactivation domain (TA), followed by a proline-rich region (PR), a central DNA-binding domain (DBD), and a C-terminal oligomerization domain (OD). In p63 and p73 there is an additional C-terminal sterile-α motif (SAM). Family members have limited overall homology, but strong similarity in the DBD (approximately 60% between p53 and p63/p73 and approximately 85% between p63 and p73).[10,138] All p53-family proteins produce two groups of mRNAs controlled by separate promoters (P1 and P2), encoding proteins with alternative N-terminal regions. Those generated from P1 promoters contain the complete TA domain and are transcriptionally proficient; those generated from P2 promoters lack the TA and are transcriptionally inactive (ΔN isoforms). For both p53 and p73, additional N-terminal variants are generated by alternative splicing or internal initiation of translation.[138] ΔN isoforms can still bind to DNA, and can exert an effect as dominant-negative versions.[138] However, this general assumption requires some caution since for instance, ΔNp63 isoforms are transcriptionally proficient.[139,140] All p53-family transcripts are also subject to alternative splicing at the C-terminus, independent of the promoter used, thus generating a combinatorial variety of isoforms (obtainable adjoining each N-terminal variant with any of the C-terminal variants). The longest C-terminal variant is dubbed α in p63 and p73, and comprises the SAM domain. In p53, the longest C-terminal isoform corresponds to the 'classic' p53 transcript, and is simply indicated as 'p53'. Alternative splicing of C-terminal exons generates shorter isoforms, named β and γ . Additional C-terminal splice variants in p73[138] are not reported in this study. The p53-family proteins also share some common post-translational modifications, reported schematically in the drawings in their respective positions; a question mark indicates that, although the modification has been shown, the target residue is unknown. It is evident that many modifications are specific for selected isoforms

p53 is a powerful tumor suppressor, as proven by a wealth of *in vivo* models and dramatically confirmed by frequent mutation in human cancers (see Hainaut *et al*.[16]Donehower and Lozano[17] and references therein). The role of the other two p53-related proteins in tumor suppression is less obvious, because they are rarely deleted or mutated in cancer, and the respective knockout mice die tumor-free from developmental defects.[2–4] Moreover, human syndromes with p63 mutations are not associated with higher tumor incidence.[18] Nonetheless, a wealth of data show that both p63 and p73 have a role in tumor suppression. First of all, a careful analysis of the tumor predisposition of p63 and p73 heterozygous mice revealed a consistent connection with cancer. In fact, p63 +/– and p73 +/– mice develop spontaneous tumors, and show a median survival time that is only a few months longer than that of p53 +/– mice.[19] Second, a number of studies showed that TAp73 and TAp63 can induce cell-cycle arrest, senescence, DNA repair, and apoptosis in response to chemotherapic drugs, independently of p53.[20–22] Third, silencing of p73 and p63 increases the transforming potential of p53–/– mouse embryonic fibroblasts.[23] Fourth, even if not mutated, p63 and p73 are aberrantly expressed in cancer. In particular, ΔN isoforms of p63 and p73 are frequently overexpressed in a wide range of tumors, in which they associate with poor prognosis (reviewed in Deyoung and Ellisen[24]). Moreover, forced expression of ΔNp73 promotes transformation in experimental models.[25,26] Thus, upregulation of ΔNp63 or ΔNp73 isoforms may be a common mechanism to inactivate the respective TA isoforms during tumorigenesis.

Recently, generation of a mouse model with selective knockout of the TAp73 isoforms, with retention of ΔNp73 expression, conclusively established a role of p73 in tumor suppression.[27] Developmental abnormalities are less severe in these mice, allowing detection of two important phenotypes: increased tumor susceptibility and infertility. Both are ascribable to increased genomic instability and aneuploidy linked with mitotic spindle defects. Thus, maintaining genome integrity seems to be a key function of TAp73.[27,28] Similarly, generation of a mouse model selectively lacking TAp63 isoforms uncovered a function for TAp63 in DNA damage-induced apoptosis of germ cells.[29] In response to genotoxic stress, TAp63 is phosphorylated by cAbl and induces apoptosis of oocytes, having a crucial role in genome protection of the female germ-line.[29,30] A more recent study established a function of TAp63 in mediating Ras-induced senescence and preventing tumorigenesis *in vivo*, in a model of p53-nullizygous mice.[20] In addition, TAp63 has a crucial role in preventing invasiveness and metastasis of epithelial tumors by controlling expression of a crucial set of metastasis-inhibitor genes.[31] Finally, it should be considered that oncogenic p53 mutants can directly or indirectly interact with p73 and p63, interfering with their functions, to promote transformation, chemoresistance, and metastasis.[31,32]

Taken together, these evidences implicate all p53 family members in tumor suppression, yet highlighting their specificities. In addition to intrinsic structural and biochemical differences, functional specificity of p53-related proteins may derive from selective interaction with specific regulators, or from interaction with common regulators that have different effects on each family member (or isoform). In any case, the settings in which p53-family proteins are activated can profoundly affect the response of diverse cell types to oncogenic stress.

Protein Interactions Modulate the Response of the p53 Pathway

The tumor suppressive function of the p53 pathway resides largely in its capacity to sense potentially oncogenic stress conditions, and coordinate a complex set of molecular events leading to growth restraining responses. All steps of this pathway are controlled by regulatory interactions with positive or negative modulators, promoting p53 covalent modifications, controlling its stability and subcellular localization, determining its specificity for selected promoters, or modulating its transactivation potential. Other p53 interactors exert an effect further downstream to directly mediate p53 biological effects, for example, at the mitochondria. Accordingly, p53 is a highly connected protein and can form physical complexes with many cellular proteins. A search of public protein–protein interaction databases using the APID web interface (http://bioinfow.dep.usal.es/apid/index.htm) currently retrieves more than 300 reported interactions involving human p53, whereas dramatically fewer interactions are reported for the other family members (Figure 2b). Such unbalance reflects a tremendous disproportion in the number of screenings conducted so far, and must not to be interpreted as a reduced propensity of p73 or p63 to form complexes with other proteins. We believe that the list of p73/p63 interactors will steadily grow as additional screens are performed.

Interactors Regulating PTM of p53-family Proteins

One important category of interactors is represented by enzymes that apply covalent PTMs on p53- and p53-related proteins, either activating or inhibiting their activity. The complex array of modifications of p53, and how these modulate its activation and functions, have been extensively reviewed recently and will not be discussed in this study (e.g., Kruse and Gu[5] and Vousden and Prives[6]). Rather, we wish to point out that several PTMs are common to multiple p53 family members (see Figure 1 and Table 1), thus implying that p53, p63, and p73 are potentially responsive to a similar set of signals. Nonetheless, the same PTM may have different effects on the biochemical functions of each p53 family member, and a given upstream signal may result in a different biological output, depending on relative expression levels of the three p53-family proteins and their isoforms. A comprehensive knowledge of regulatory modifications of all three p53-related proteins will improve our understanding of the behavior of the p53 pathway in different cell types, and perhaps allow development of better prognostic and therapeutic strategies.

Not too much, not too little: Interactors Regulating p53-family Protein Levels

The principal way in which p53 levels are controlled is by regulated degradation of the protein. Accordingly, ubiquitination of p53 represents a 'core control' on which many input signals converge. Perhaps less prominent is the role of protein turnover in the regulation of p73 and p63, but a number of ubiquitin ligases are shared among p53 family members.

MDM2. The most important p53 regulator is the Ring domain E3 ubiquitin ligase MDM2 (mouse double minute-2). MDM2 recognizes a short region in the TA domain of p53 and interferes with its transcriptional activity; at the same time, MDM2 interacts with the DBD region and ubiquitinates p53, promoting its proteasomal degradation.[33,34] As MDM2 is a transcriptional target of p53, inhibition by MDM2 is part of a negative feedback loop on p53 activation.[34] In addition to MDM2, p53 is also bound and regulated by the MDM2-related protein MDMX (also named MDM4). MDMX does not induce p53 degradation directly, but can antagonize p53-dependent transcription.[35] However, MDMX and MDM2 heterodimerize to augment p53 degradation, and hence both proteins can potentially influence p53 stability.[36,37]

Both MDM2- and MDMX-deficient mice die *in utero* as a consequence of p53 hyperactivity, showing the crucial role of these proteins in restraining p53 function during development.[38,39] Not surprisingly, high levels of MDM2 or MDMX are found in many human cancers.[40,41]

MDM2 and MDMX also bind p73, but MDM2 does not promote p73 ubiquitination.[42,43] Rather, MDM2 relocalizes p73 to subnuclear speckles and represses p73 transcriptional activity by preventing its interaction with the acetyltransferase p300/CBP and RNA polymerase-associated factors.[44,45] Interestingly, MDM2 can also catalyze addition of the small ubiquitin-like protein NEDD8 to both p53 and p73, and also this modification inhibits their transcriptional activity.[46,47]

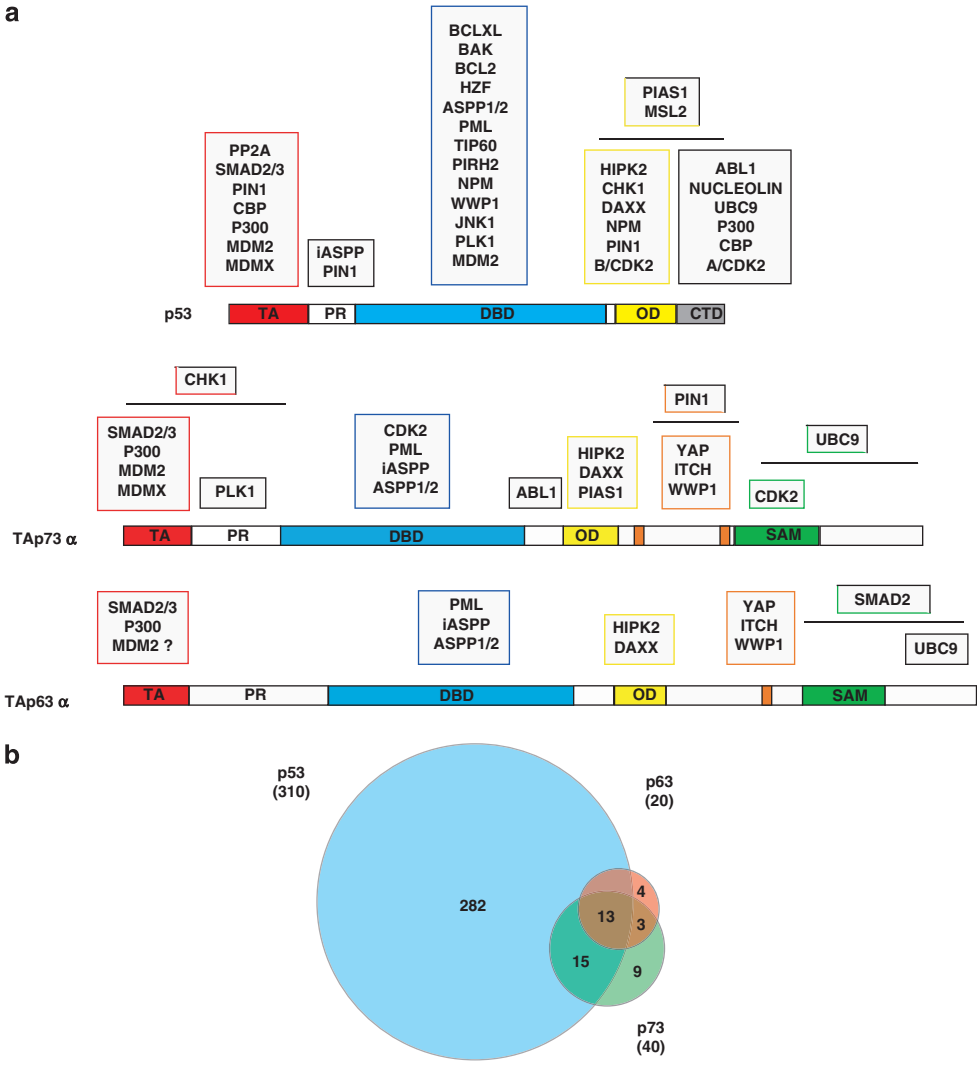

Figure 2 Protein–protein interactions of the p53 family. (**a**) The figure summarizes some interactors of the p53-family proteins, arranged according to their region of binding. The figure reports also proteins that are not mentioned in this review, but for which the region of interaction has been reliably mapped. Horizontal bars indicate that the interaction has not been mapped in greater detail. Orange vertical boxes mark the PY motif (the sequence PPxY) present in p73 and p63. (**b**) Venn diagram summarizing protein interactions currently associated with the three p53 family members in mammals. Data were retrieved using the APID web interface (http://bioinfow.dep.usal.es/apid/index.htm) and manually amended according to the most recent literature

Less is known about the physical association of MDM2 and MDMX with p63. Some researchers found lack of interaction for both MDM2 and MDMX,[48,49] whereas others were able to detect MDM2–p63 complexes, although reporting contradictory effects on p63 function.[42,50] The crucial amino acids required for MDM2 binding are conserved in p63, but molecular modeling suggests that other residues in the N-terminal domain of p63 may render this interaction significantly weaker.[51]

In line with its role as primary regulator of p53, MDM2 stability, localization, and function are tightly controlled, and hence the p53–MDM2 core circuit responds to a multitude of signaling pathways, including DNA damage, oncogene activation, and nucleolar/ribosome stress (see Kruse and Gu[5], Vousden and Prives[6] and references therein). Similarly, stress-activated kinases can regulate MDMX activity.[52]

In addition to stress, the p53–MDM2 core circuit can also be regulated by physiological cues. An example is the Notch pathway, crucial for determination of cell fate, maintenance of stem-cell populations, and often deregulated in cancer. p53 can inhibit Notch signaling by both repressing transcription of Presenilin 1 and competing with Notch for interaction with the transcriptional co-activator MAML1.[53] At the same time, Notch can negatively regulate p53 by modulating the p53–MDM2 core circuit; in fact, Notch1 signaling increases MDM2 activity through downregulation of p14ARF and activation of the PI3K/Akt pathway.[54] In line with this antagonism, the endocytic protein Numb, which is asymmetrically partitioned at mitosis to control cell fate by antagonizing the activity of Notch, binds to both MDM2 and p53, counteracting MDM2-dependent p53 ubiquitination and promoting p53 stabilization and activity.[55] The p53–MDM2 core circuit also mediates a functional link between p53 and the Hedgehog signaling

Table 1 A selection of enzymes that apply post-translational modifications on p53-family proteins, or were reported to physically interact with them, independently of modification

	p53	p73	p63	References[a]
Kinases				
ABL1	+	+	+	30, 67–69
ATM	+		+	122
CDK2	+	+	+	122, 123
CHK1	+	+		124
CK1	+			
GSK3-beta	+		+	125
HIPK2	+	+	+	96
JNK1	+	+		126
p38	+	+	+	100, 127
PLK1	+	+		128
Phosphatases				
PP2A	+		+	125
Wip1	+			
Acetyltransferases				
P300/CBP	+	+	+	77, 78, 100
PCAF	+	+		77
TIP60	+	+		129
De-acetylases				
HDAC1/2	+	+		130
Sirt1	+	+		83
Ubiquitin ligases				
MDM2	+	+	+ /–	44, 49, 50
MDMX	+	+	–	42, 43, 49
COP1	+			
PIRH2	+			
Synoviolin	+			
ARF-BP1	+			
CHIP	+			
WWP1	+	+	+	63, 64
ITCH	–	+	+	65, 66
E4F1	+			
TRIM24	+			
FBXO45	–	+		62
Deubiquitinases				
HAUSP	+			131
USP10	+			
Methyltransferases				
Smyd2	+			
SET7/8	+			
PRMT5	+			
Demethylases				
LSD1	+			
SUMO ligases				
Ubc9	+	+	+	132, 133
PIAS1	+	+		134
TOPORS	+			
Others				
O-GlcN-Ac transferase	+			
PARP-1	+			
PIN1	+	+		73

+, Reported modification and/or interaction; –, reported experimental evidence of lack of interaction. [a]Only evidences regarding p73 and p63 are referenced. Most regulatory modifications of p53 have been reviewed elsewhere[5,135–137]

pathway. In fact, constitutive activation of the Hedgehog pathway downregulates p53 by promoting MDM2 phosphorylation and association with p53.[56]

As MDM2 binds multiple p53 family proteins, the many signaling pathways that impinge on the MDM2–p53 core circuit could in principle engage the entire p53 family in a coordinated cellular response.

Other E3 ubiquitin ligases. In addition to MDM2, several other ubiquitin ligases promote p53 degradation (see Table 1). Two such ligases, PIRH2 and COP1, are direct transcriptional targets of p53; together with MDM2, they are involved in a negative feedback loop to restore normal p53 levels after the stress response. On the contrary, several other E3 ubiquitin ligases can regulate p53 turnover in the absence of stress (see Lee and Gu[57] and references therein). Notably, MDM2 and MDMX are found only in vertebrates, whereas most of the other p53-specific ubiquitin ligases are conserved in simpler organisms such as *Caernorhabditis elegans* and *Drosophila melanogaster*. Currently, little is known on the potential activity of these ubiquitin ligases on Cep-1 or Dmp53 (p53-like proteins of *C. elegans* and *D. melanogaster*, respectively). For example, the cytoplasmic ubiquitin ligase Synoviolin degrades both mammalian and *Drosophila* p53.[58] Similarly, TRIM24, a RING-domain ubiquitin ligase that degrades mammalian p53, may be controlling p53 levels in *Drosophila,* as the TRIM24 knockout phenotype is rescued by knockdown of Dmp53.[59,60] Considering that invertebrates have a single p53-like gene, it is possible that evolutionarily conserved ubiquitin ligases that degrade invertebrate p53 can bind and modulate p63 and/or p73 in mammals. A proof of principle is set by FBXO45, the mammalian ortholog of *C. elegans* FSN1, an F-box protein that is involved in germ-line apoptosis by regulating the p53-like protein Cep-1.[61] In mammalian cells, FBXO45 binds specifically to p73 and promotes its degradation by an SCF-dependent mechanism.[62]

WWP1. p53-family proteins are also regulated by NEDD4-like ubiquitin ligases, characterized by one or more WW domains that recognize proline-rich sequences on target proteins. The ubiquitin ligase WWP1 binds a PY motif (the PPxY sequence) in the C-terminal region of p63, and induces its ubiquitin-dependent proteasomal degradation.[63] The presence of a conserved PY motif in p73 may be sufficient to predict a similar activity of WWP1 on this protein. Currently, binding and ubiquitination of p73 by WWP1 have been only reported as unpublished data.[63] Very intriguingly, despite the lack of a canonical PY motif, WWP1 can also bind p53; this interaction produces a mono-ubiquitinated form of p53 that is retained in the cytoplasm, inhibiting its transcriptional activity.[64] WWP1 is overexpressed in a significant fraction of breast and prostate tumors, and its activity on multiple p53-family proteins suggests a potential role in oncogenesis.

ITCH. The NEDD4-like ubiquitin ligase, ITCH, binds p73 and p63 through interaction with the C-terminal PY motif, and promotes their proteasomal degradation. In contrast to WWP1, it does not interact with p53.[65,66] Both TAp73 and TAp63 accumulate after DNA damage,[30,67–69] and stabilization of TAp73 and TAp63 in response to stress may involve inhibition of ITCH-mediated ubiquitination.[65,66] In contrast to the MDM2–p53 core circuit, stress signals apparently do not dissociate the complex between ITCH and

p73 directly. Rather, ITCH is downregulated upon stress, through a mechanism still poorly understood.[66] A recent paper uncovered the involvement of miRNA-106b in translational repression of ITCH, showing re-activation of p73-dependent apoptosis in primary chronic lymphocytic leukemia cells after miR-106b induction.[70] Taken together, these evidences point to ITCH as a major regulator of p73 cellular levels.

The p73–ITCH regulatory circuit is modulated by the adaptor protein YAP-1 (Yes-associated protein 1) that binds p73 through the same PY motif as Itch and prevents Itch-mediated p73 degradation.[71] Accordingly, association with YAP has a crucial role in controlling p73 levels after DNA damage.[71] Interestingly, YAP also promotes interaction between p73 and the p300 acetyltransferase, with implications on p73 transcriptional activity.[72] As p300-dependent acetylation is also linked with p73 stability,[73] YAP-dependent p73–p300 association could be functional first for p73 stability, and then for p73 transcriptional activity. The adaptor YAP is also a direct target of the PI3K/Akt signaling pathway,[74] which has a central role in growth and proliferation in normal cells and in tumors, contributing to cell survival by blocking apoptosis. Notably, this pathway intercepts both p53 and p73, as Akt-dependent phosphorylation of MDM2 enhances its nuclear accumulation and thus promotes p53 degradation,[75] whereas phosphorylation of YAP interferes with its nuclear import and thus prevents p73 stabilization.[74] YAP also binds p63,[76] but it is still unknown whether YAP can interfere with ITCH-mediated p63 turnover similarly to what is described for p73. If this was the case, YAP would qualify as a central modulator of p63/p73 biological activities uncoupled from the p53 response.

Interactors Regulating Transcriptional Functions of the p53 Family

All p53-family proteins are transcription factors, and the main output of the p53 pathway is the coordinated transcriptional regulation of a wide array of cellular genes. Therefore, a great deal of interest is focused on understanding how different sets of genes are regulated by p53-family proteins in a signal- and context-dependent manner. Promoter selection has an integral part in determining the response to p53 family members, and differences in the sequence and spacing of p53-binding sites, specific PTMs, together with the presence or absence of specific cofactors, all contribute to promoter selection and, in turn, cellular response (see Vousden and Prives[6] and Menendez *et al*.[8] and references therein).

Modulating transactivation. A well-defined mechanism contributing to p53-dependent transactivation is the ability to recruit chromatin remodeling complexes and histone modifiers on target promoters. p300/CBP (CREB-binding protein) as well as pCAF acetyltransferases bind to p53 and promote transcriptional activity catalyzing acetylation of lysines within the p53 C-terminal region.[5] Although p53 modification may be necessary for the recruitment of TAFs (transcription-associated factors), the concomitant induction of histone acetylation contributes to the open status of the chromatin.

Both p73 and p63 are also bound by p300/CBP, promoting their transcriptional activity, and hence this regulation is highly conserved.[77,78] In line with this evidence, phosphorylation-dependent Pin1-mediated prolyl-isomerization stimulates p73 acetylation by p300/CBP, and is required for p73-dependent apoptosis in response to DNA damage.[73]

The importance of acetylation for p53 function is questioned by the observation that knock-in mice with all C-terminal lysines mutated to arginine have mild phenotypes, suggesting that PTMs of the C-terminal lysines, including acetylation, are not crucial for p53 function *in vivo*.[40] However, this contradiction might be solved by the recent identification of two additional acetylation sites in the core domain of p53 that affect its transcriptional activity: K164 (modified by p300/CBP and pCAF) and K120 (modified by Tip60).[79,80] Moreover, the evidence that deacetylases such as HDAC1/2 and SIRT1 can inhibit p53 and p73 transcriptional activity[81–83] keeps alive the debate on the importance of acetylation for p53-family proteins.

JMY. Specific cofactors can modulate the interaction between p53 and acetyltransferases. One interesting example is JMY (junction-mediating and regulatory protein), a cytoplasmic actin-binding protein that can promote microfilament polymerization and directly interacts with p53 and p300/CBP.[84–87] After DNA damage, JMY accumulates in the nucleus, in which it associates with p53 and the tetratricopeptide repeat-containing protein STRAP to form a complex that includes p300/CBP, and promotes p53-dependent transcription and apoptosis.[84,87] Interestingly, STRAP can also recruit within this complex the arginine methylase PRMT5 that modifies three residues in the C-terminal region of p53.[88] Modification of p53 by the JMY-STRAP-PRMT5-p300/CBP complex stimulates transactivation of the p21Waf1 promoter, shifting the p53 response toward cell-cycle arrest rather than apoptosis.[88] As all p53-family proteins are regulated by acetyltransferases, it is legitimate to predict the existence of analogous regulatory complexes to control association of p63 and p73 with p300/CBP or other post-translational modulators. Research in this field should be encouraged.

PML. Association with regulatory cofactors is also controlled by interaction with scaffolding proteins and accumulation in specific compartments. Perhaps the best example of such regulation is the interaction of p53, p63, and p73 with the promyelocytic leukemia protein (PML). In fact, all three p53-family proteins can be recruited to subnuclear structures called PML nuclear bodies (PML-NB), and association with PML promotes their modification, stabilization, and activation.[72,89,90] A number of common interactors of p53-family proteins are also found in PML-NBs, including the acetylase p300, the protein kinase Hipk2, and the transcriptional repressor Daxx, and hence PML-dependent recruitment to NBs might regulate formation of specific protein complexes. Notably, PML facilitates the interaction of YAP with p73 (see above), stimulates the recruitment of p300 on p73, and promotes p73-dependent transactivation of pro-apoptotic target genes after DNA damage.[72] Therefore, PML may add a further level of modulation to the p73–ITCH–YAP regulatory

loop. Intriguingly, both p73 and p53 directly regulate transcription of PML, which in turn facilitates their modification and promotes their functions, generating a positive autoregulatory feedback loop.[72,91]

Axin. Another example of a scaffolding protein involved in p53 regulation is Axin, a component of the canonical WNT pathway that also binds Daxx and protein kinase Hipk2.[92] A recent study uncovered that protein–protein interactions assisted by the Axin scaffold actively contribute to determine the extent of p53 phosphorylation on Ser46, thus controlling cell fate in response to different levels of DNA damage.[93] It should be noted that p73 and p63 also interact with Daxx[94,95] as well as with Hipk2,[96] and therefore the role of Axin in controlling p53 modification by Hipk2 may extend to the other family members.

Determining target gene selection. The mechanism regulating the selective binding of p53-family proteins to different promoters under different conditions is one of the most intriguing open questions in the field. In some cases, the binding of specific interactors with p53, p63, or p73 has been shown to influence the choice of target promoters, resulting in the induction of cell-cycle arrest or apoptosis.

ASPP proteins. The evolutionarily conserved ASPP family (Ankirin repeats, SH3 domain, proline-rich protein) regulate the pro-apoptotic, but not cell-cycle functions of all p53 family members. The ASPP1/2 proteins bind p53 and increase transcription of pro-apoptotic genes such as Bax and PIG3.[97] Chromatin IP experiments revealed that interaction with ASPP1/2 promotes the selective recruitment of p53 on the promoters of BAX and PIG3, but not p21WAF or MDM2.[98] Similarly, the ASPP1/2 proteins interact with the other p53 family members, p73 and p63, and also stimulate transactivation of pro-apoptotic gene promoters.[97]

On the other hand, the inhibitory protein iASPP binds p53 and inhibits its ability to upregulate pro-apoptotic genes, without affecting transcription of genes mediating cell-cycle arrest.[99] Accordingly, dissociation of p53 from iASPP is facilitated by Pin1-catalyzed prolyl-isomerization after phosphorylation of Ser46, and contributes to p53 activation in response to lethal DNA damage.[100] Notably, iASPP also binds and inhibits p73; expression of a peptide displacing the iASPP-p73 interaction was shown to promote p73-dependent apoptosis in transformed cells lacking p53.[101]

Therefore, cellular levels of ASPP proteins, differentially affecting all p53-family proteins, may be a crucial parameter in determining the apoptotic readout of the p53 pathway. Not surprisingly, altered expression of ASPP genes is a frequent event in tumors.[102] As ASPP proteins have different binding affinities for distinct p53 family members,[103] the development of competitor peptides potentially displacing iASPP from p53 and/or p73 may become a promising strategy to modulate ASPP functions in tumors.

Cabin1. Another interesting example is Cabin1, a protein that modulates p53 transcriptional activity on selected target gene promoters. Under normal conditions (i.e., in the absence of stress) p53 occupies a subset of its target promoters such as Gadd45, p21Waf1, Puma, Noxa, and MDM2. With the exception of the MDM2 promoter, Cabin1 colocalizes with p53 on these sites, recruiting histone deacetylases and methyltransferases to make chromatin unsuitable for transcription. After DNA damage, Cabin1 is rapidly degraded, allowing promoter-bound p53 to induce an immediate transcriptional response.[104] Therefore, Cabin1 functions as a rather fast transcriptional switch on selected p53 promoters. As Cabin1 binds the core DBD region of p53, which is the most conserved among p53-family proteins, it will be interesting to test whether a similar mechanism may also apply to promoters bound and regulated by p73 and p63.

Smads. The choice of what genes are induced or repressed by p53 is also determined by cooperation with other transcription factors that bind discrete responsive elements in target promoters. One example of such regulation is the interaction of p53-family proteins with receptor Smads (R-Smads), intracellular transducers of TGF-β signaling.[105] p53 binds Smad2 and Smad3, and cooperates synergistically with Smads to regulate transcription of a subset of TGF-β target genes. More specifically, Smad2/3 and p53 bind on two separate adjacent responsive elements in the promoter of the activin-responsive gene Mix.2.[105] An alternative architecture has been described in human α-fetoprotein (AFP), in which a single Smad/p53-responsive element is occupied simultaneously by both proteins; in this case, p53 interaction with Smad2 and SnoN represses AFP transcription.[106] Importantly, all three p53-family proteins interact physically and functionally with R-Smads, and can cooperate with Smad2/3 to regulate transcription of a Mix.2 reporter construct in response to TGF-β.[31,105] Therefore, joint control of target genes by TGF-β and p53-family proteins may be a widespread mechanism.

The Nucleus is not Enough: Interactors Mediating Cytoplasmic Functions of the p53 Family

In addition to the well-established transcriptional functions, there are several evidences for transcription-independent tumor suppressive roles of p53 in the cytosol. The key to these functions is regulation of p53 subcellular localization. One of the major mechanisms for p53 cytosolic relocalization is MDM2-mediated monoubiquitination, which does not induce p53 proteasomal degradation but nuclear export.[107] The E3 ligases WWP1 and MSL2 are also involved in p53 nuclear export, indicating that ubiquitination is a pivotal mechanism for p53 cytosolic localization.[108] Notably, in some instances ubiquitination can have the opposite effect. In fact, the zinc-finger protein E4F1 binds p53 and promotes its ubiquitination on an atypical lysine residue; this modification does not induce nuclear export but stimulates p53 recruitment on chromatin and expression of a subset of target genes.[109]

Another mechanism affecting nucleo-cytoplasmic localization of p53 is interaction with and modification by PARP-1, a poly-ADP-ribose polymerase activated by DNA damage; ADP-ribosylation blocks nuclear export of p53 and increases its transcriptional activities.[110] These evidences

emphasize that p53 cytosolic localization is a finely regulated phenomenon.

Dramatically less is known about cytoplasmic localization of the other p53 family members, although evidences for non-nuclear roles of these proteins are beginning to accumulate (see below); in this regard, it would be interesting to explore whether monoubiqitination or alternative types of modification are actually conserved in p63 and p73.

The best-characterized non-transcriptional function of p53 is the induction of apoptosis through the mitochondrial pathway. After DNA damage or oncogene activation, mono-ubiquitinated p53 localizes to the mitochondria; there, it interacts with a mitochondrial pool of the deubiquitinase HAUSP that removes ubiquitin and allows p53 to form complexes with BCL2, BCL-xL, BAX, and BAK, thereby promoting apoptosis.[7,111] An unexpected role of MDMX/MDM4 in p53-mediated mitochondrial apoptosis has also been recently described.[112] MDMX can localize to the mitochondria and associate with BCL2 in normal growing cells; under cytotoxic stress conditions, MDMX exerts an effect as mitochondrial anchor for p53 phosphorylated on Ser46, and favors p53–BCL2 interaction to trigger the intrinsic apoptotic pathway.[112]

Of note, Hipk2 (one of the kinases responsible for p53 phopshorylation on Ser46) and MDMX are shared interactors of all p53-family proteins. Moreover, p53 binds BAK, BCL-2, BAX, and BCL-xL through the DNA-binding domain, which is the region of higher similarity among family members. Therefore, it would not be surprising if p73 and p63 will also prove to be involved in the intrinsic apoptotic pathway. Indeed, it has been recently reported that p73 is cleaved by caspase-3 and caspase-8 in cells undergoing apoptosis after death-receptor activation by TRAIL (TNF-related apoptosis-inducing ligand). Under these conditions, caspase-generated p73 fragments localize to mitochondria and contribute to TRAIL-induced apoptosis.[113] Full-length p73 was also detected in mitochondria by biochemical fractionation and electron microscopy. Most importantly, addition of recombinant TAp73 to purified mitochondria induced mitochondrial outer membrane permeabilization, suggesting that p73 has non-transcriptional pro-apoptotic functions analogous to those of p53.[113]

Another emerging non-nuclear function of p53 is in regulation of autophagy,[114] a process that allows removal of damaged cytoplasmic organelles and adaptation of cells to metabolic stress. Although p53 can transactivate genes that induce autophagy under stress conditions (e.g, DRAM, TSC2, Sestrin1 and 2, PTEN, and IGFBP3), depletion or mutation of p53 actually *increases* autophagy, suggesting that p53 constitutively limits this process in normal growing cells.[7,115,116] Even if the mechanism remains unknown, the autophagy-inhibitory activity is ascribable to the cytoplasmic pool of p53, as degradation of cytosolic p53 by MDM2 promotes autophagy after nutrient depletion, endoplasmic reticulum stress, or treatment with rapamycin.[114] Besides

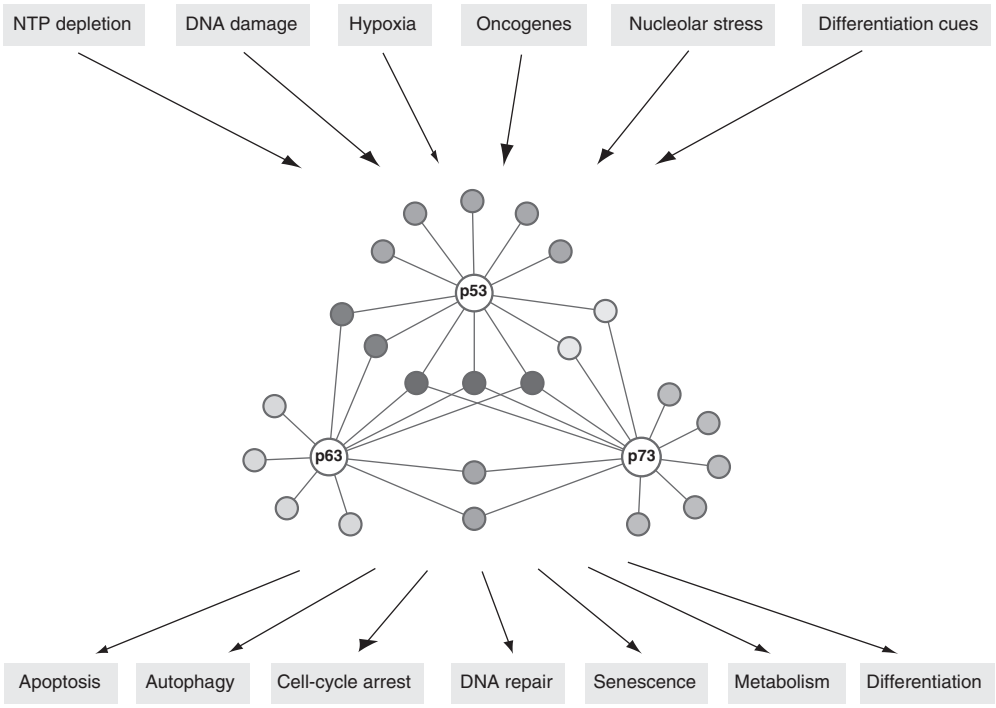

Figure 3 The p53 family as a network. The p53-family pathway is activated by a wide array of signals, including potentially oncogenic stresses, as well as physiological cues. Once activated, the pathway induces diverse cellular outcomes, ranging from cell-cycle arrest, to senescence, to programmed cell death (apoptosis). A web of upstream regulators control covalent modifications, protein levels, and cellular localization of p53-family proteins, and can either activate or inhibit the pathway in response to specific signals. Downstream, protein cofactors modulate promoter selection and transcriptional functions of p53-family proteins, fine-tuning the cellular response to any given signal. In addition, protein interactors can behave as direct effectors of non-transcriptional functions of p53-family proteins, for instance, in mitochondrial apoptosis. Some protein modulators are specific for each p53-related protein, whereas others are shared among the p53 family. Moreover, common interactors may have similar or different effects on each p53-family protein. As a result, the cellular outcomes to any given conditions are influenced by the expression levels of each p53 family member, as well as by the pattern of modulators that are expressed in a given cell or tissue, and their respective expression levels

MDM2, additional ubiquitin ligases can mediate cytosolic p53 degradation; for example, the already mentioned Synoviolin.[58] Interestingly, a recent study revealed that p300/CBP can also function as ubiquitin ligases for cytoplasmic p53.[117] As p300 and CBP can also interact with p73 and p63, it will be interesting to explore whether their ubiquitin ligase activity may extend to all p53 family members. In any case, the potential role of these cytoplasmic ubiquitin ligases in autophagy remains to be investigated.

The evidence of important non-nuclear roles of p53 (and perhaps p73) imposes a careful analysis of the cytosolic life of this family of proteins. In fact, a better knowledge of the mechanisms controlling their subcellular localization and their connections with other signaling pathways may offer new opportunities to better define the 'p53 system' and improve its pharmacological modulation.

The p53 Pathway as a Network

This overview of some regulatory interactions of the p53 family highlights the concept that multifaceted aspects of this pathway are dependent on a wide repertoire of protein–protein interactions that, although often shared among the family members, can have similar or different effects on each p53-family protein.

Considering the complex network of protein interactions modulating all p53-family proteins, the whole family should be considered when analyzing the genetics of cancer cells (Figure 3). Fluctuations in the levels of selected p53 family members (or their isoforms) might change the relative availability of shared protein partners, as multiple p53-family proteins compete for interaction. Also, differential expression of selected interactors – linked with genetic variation, for example – may distinguish the response of the p53 pathway to the same potentially oncogenic stimuli in diverse individuals.

In tumor cells, loss of p53 abrogates one very crucial node of this network, but p73 and p63 may compensate to some extent. In contrast, mutation of p53 (mut-p53) generates a dominant protein, often very stable, that can still form complexes with a subset of p53 partners, and may also acquire novel interactors. High levels of mut-p53 may be sufficient to bind and inhibit p73 and p63, as well as to titrate common interacting proteins, thus making the network extremely weak. For this reason, displacing mut-p53 interactions may become a promising approach to combat the more aggressive features of tumors bearing p53 mutations.[118,119] Finally, functional loss or deregulated expression of some crucial p53 interactors are frequent in primary cancers and transformed cell lines bearing wild-type p53 (e.g., p14ARF, MDM2, Chk2, iASPP etc.); these alterations may have a greater impact if they affect regulators that are common to all p53-family proteins (e.g., MDM2 or ASPP proteins), with relevant implications for cancer prognosis and, eventually, therapy. In our opinion, these premises are sufficient to promote the systematic study of the protein interaction profile of the entire p53 family.

p53 has recently turned 'thirty'. During these years, a p53-centric approach to the pathway has yielded some very promising therapeutic tools, including small molecules that target the MDM2–p53 interaction (e.g., Nutlin[120] and RITA[121]). However, our awareness of the complexity of the p53 pathway has grown significantly, and we should no longer think of p53 as a 'lone warrior' in tumor suppression. For this reason, it will be important to develop more reagents and experimental tools to study all p53-family proteins, and their isoforms, at a greater level of resolution. Such knowledge will allow a more efficient use of currently available drugs, and will increase the number of regulatory interactions that may become potential targets for new therapeutic molecules.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements. We apologize to many colleagues whose contributions have not been described, or even cited, due to space constraints. We acknowledge support from AIRC (Italian Association for Cancer Research) to GDS and LC, and EC FP6 contracts 503576 and 502983 to GDS.

Note added in proof

We recently identified several additional potential interactors of p53-family proteins that were not mentioned in this review. Lunardi A, Di Minin G, Provero P, Dal Ferro M, Carotti M, Del Sal G *et al*. A genome-scale protein interaction profile of Drosophila p53 uncovers additional nodes of the human p53 network. *Proc Natl Acad Sci USA* 2010. [E-pub ahead of print 22 March 2010].

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