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p53 Isoforms: Key Regulators of the Cell Fate Decision

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It is poorly understood how a single protein, p53, can be responsive to so many stress signals and orchestrates very diverse cell responses to maintain/restore cell/tissue functions. The uncovering that *TP53* gene physiologically expresses, in a tissue-dependent manner, several p53 splice variants (isoforms) provides an explanation to its pleiotropic biological activities. Here, we summarize a decade of research on p53 isoforms. The clinical studies and the diverse cellular and animal models of p53 isoforms (zebrafish, *Drosophila*, and mouse) lead us to realize that a p53-mediated cell response is, in fact, the sum of the intrinsic activities of the coexpressed p53 isoforms and that unbalancing expression of different p53 isoforms leads to cancer, premature aging, (neuro)degenerative diseases, inflammation, embryo malformations, or defects in tissue regeneration. Cracking the p53 isoforms' code is, thus, a necessary step to improve cancer treatment. It also opens new exciting perspectives in tissue regeneration.

p53 is a central sensor of cell signals and a master regulator of cell response to damage (Lane and Levine 2010). But how can so many distinct extracellular and intracellular signals modulate p53 activity? How can only one protein bind specifically to so many different DNA sequences (p53 response elements) (el-Deiry et al. 1992; Bourdon et al. 1997; Khoury and Bourdon 2011; Simeonova et al. 2012) and directly regulate expression of thousands of genes? How does p53 select the target genes to trigger on time the appropriate cell responses to so many different cellular damages? Why is it so difficult to link *TP53* mutation status to prognosis and cancer treatment?

The *TP53* gene is highly conserved in multicellular organisms (Lane et al. 2010). It is located on human chromosome 17p13.1 and is composed of 13 exons of which the first is noncoding (Fig. 1A). *TP53* contains the *Hp53Int1* and *WRAP53* genes within exon-1/intron-1 (Reisman et al. 1996; Mahmoudi et al. 2009). It presents multiple genetic polymorphisms defining more than 100 distinct *TP53* haplotypes, some of which are correlated with an increased risk of cancer (Dumont et al. 2003; Garritano et al. 2010; Wu et al. 2013). Although it is unequivocally established that *TP53* is the most frequently mutated gene in human cancer, it is still difficult in the clinic to link *TP53* mutation status to cancer treatment and clinical outcome, suggesting that the p53 pathway is not entirely understood. The discovery that the *TP53* gene encodes several different splice variants may explain the discrepancy.

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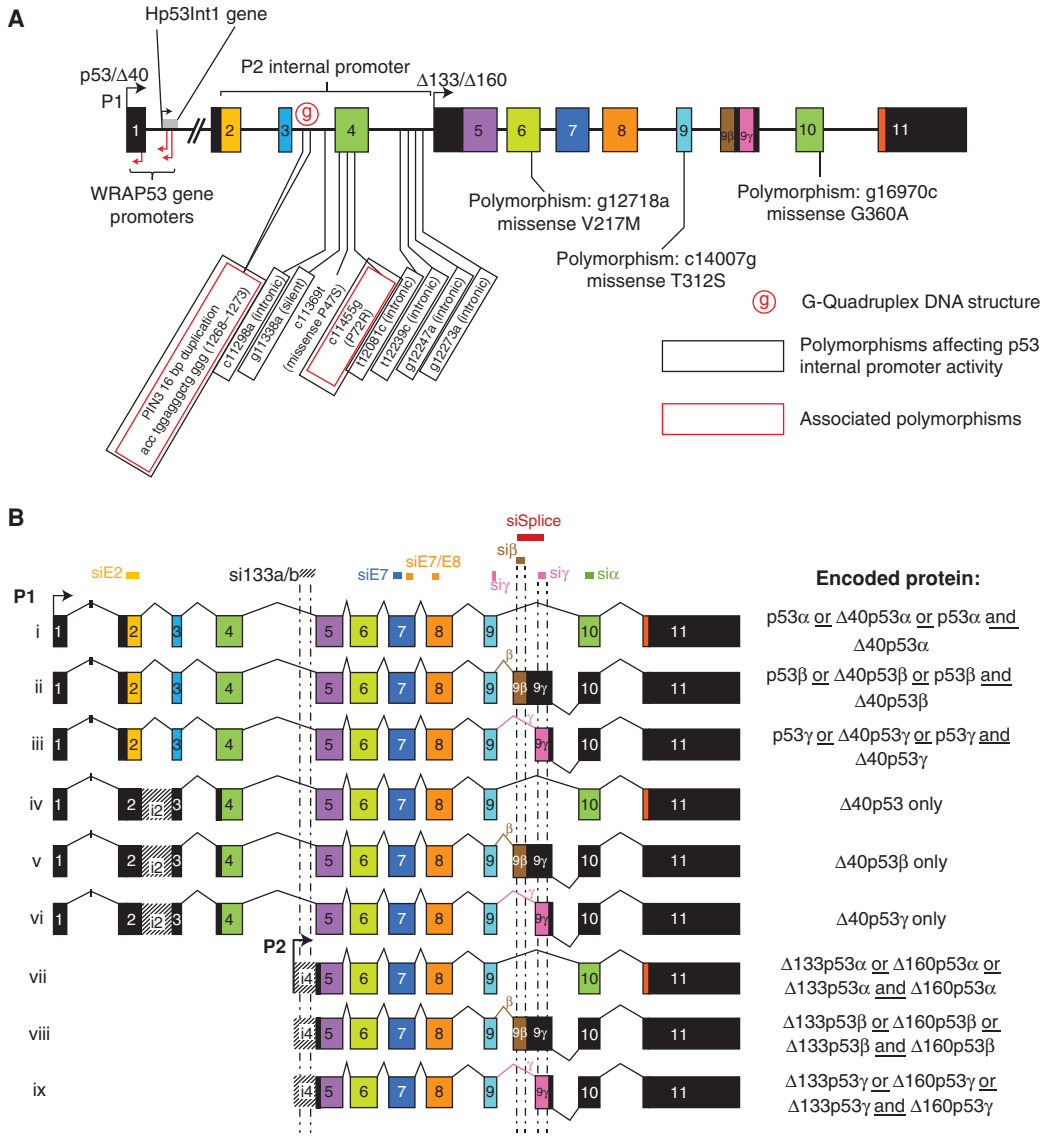

Figure 1. *TP53* locus and p53mRNAs. All introns/exons are represented to scale. Black boxes represent noncoding sequences, whereas coding sequences are colored. (*A*) The human *TP53* gene's locus structure. The *TP53* gene, which is composed of 11 exons and two cryptic exons (9β and 9γ), encodes several p53 isoforms attributable to alternative promoters (↱ P1 and P2) and alternative retention of the cryptic exons. The noncoding exon-1 and intron-1 contain different promoters for the *WRAP53* gene (antisense-coded) and intron-1 contains the *Hp53Int1* gene. A G-quadruplex DNA structure located within intron-3 modulates splicing of intron-2 and activities of the internal p53 promoter P2. Several polymorphisms (including Pin3 and R72P) change activities of the internal p53 promoter (P2). (*B*) p53 mRNAs. The *TP53* gene encodes nine different mRNAs attributable to the alternative promoters (↱ P1 and P2) and splicing (^). The promoter P1, located upstream of exon-1, encodes for intron-2 spliced (i, ii, and iii) or intron-2 retained (iv, v, and vi) mRNAs. The intron-2 spliced mRNAs can encode the full length (ATG1) and/or the Δ40 (ATG40) proteins, depending on the cell context, whereas the mRNA retaining intron-2 can only encode the Δ40 proteins. The P2 initiation transcription site is located in intron-4 and encodes for three transcripts (vii, viii, and ix), which encode the Δ133 and the Δ160 forms. Small interfering RNAs (siRNAs) targeting the different p53 isoforms are represented on top of the corresponding exons or introns.

p53 splice variants were first identified in the late 1980s in human and mouse (Matlashewski et al. 1984; Wolf et al. 1985). Thereafter, an alternative splicing of *TP53* intron-9 has been described (Arai et al. 1986; Flaman et al. 1996). To date, in human, nine p53 mRNAs (Fig. 1B) encoding 12 different p53 protein isoforms have been described (Bourdon et al. 2005; Marcel et al. 2010a), p53α (also named full-length p53, FLp53, canonical p53, TAp53α), p53β (or p53i9), p53γ, Δ40p53α (or ΔNp53, p44 or p47), Δ40p53β, Δ40p53γ, Δ133p53α, Δ133p53β, Δ133p53γ, Δ160p53α, Δ160p53β, and Δ160p53γ (Fig. 2A).

THE HUMAN p53 ISOFORMS

For decades, it was thought that one gene encodes one protein. The sequencing of the human genome changed this dogma and revealed that ~98% of the human genes are alternatively spliced and contain multiple initiation sites of transcription (promoter). The *TP53* gene is no exception. To date, it is reported that human *TP53* differentially expresses in normal tissue at least nine mRNAs in a tissue-dependent manner. They are a result of alternative promoter usage (P1 and P2) and alternative splicing of intron-2 and intron-9 (Fig. 1B). Furthermore, depending on the cell type, the translation of the p53 mRNAs can be initiated at different codons. For the mRNAs transcribed from the proximal promoter (P1), translation can be initiated at codons 1 and/or 40, whereas the mRNAs transcribed from the internal promoter (P2) translation can be initiated at codons 133 and/or 160.

The fully spliced p53 transcript (i) encodes the canonical p53 protein (p53α) but also encodes the Δ40p53α isoform thanks to an internal ribosomal entry site (IRES) (Yin et al. 2002; Candeias et al. 2006; Ray et al. 2006). This transcript also exists with two different alternative splicings of exon-9 retaining thus the exon-9β or -9γ (ii/iii) and encoding, respectively, the p53β and/or Δ40p53β, and the p53γ and/or Δ40p53γ. Both exon-9β and exon-9γ contain stop codons so that exon-10 and -11 are noncoding in β and γ p53 mRNA splice variants. The second type of transcript, also expressed from the promoter P1, conserves intron-2 (iv/v/vi). Retention of this intron leads to several stop codons when translation is initiated from codon 1, thus preventing synthesis of full-length p53 proteins. However, translation can still be initiated at codon 40 so that this group of mRNAs encodes exclusively Δ40p53α, Δ40p53β, or Δ40p53γ. The mRNAs vii/viii/ix are transcribed from the internal promoter P2 located within intron-4. Their translation can start either at the codons 133 or 160 encoding, thus, the Δ133 and Δ160 p53 protein isoforms (α, β, or γ, respectively).

At the protein level, p53α (canonical p53) was the first p53 protein isoform to be identified. It is a 393-amino-acid-long protein with seven functional domains (Fig. 2A,B). The amino terminus is composed of two transactivation (TA) domains, TA-1 and TA-2, which are required to induce a distinct subset of p53-target genes. p53α also contains a proline-rich domain (PRD), a DNA-binding domain (DBD), and a hinge domain (HD). The carboxyl terminus is composed of an oligomerization domain (OD) and a negative regulation domain (α). The negative regulation domain, rich in lysine (K-rich), undergoes many different posttranslational modifications (phosphorylation, methylation, acetylation, sumoylation, ubiquitinylation, neddylation, etc.), which regulate p53α activity and stability (Meek and Anderson 2009). In addition, the hinge and α domains contain several nuclear localization signals (NLSs). The p53 DBD protein sequence is highly conserved through evolution. It contains several conserved cysteines and histidines that coordinate Zn^{2+} or Mg^{2+} essential for p53 conformation and DNA-binding activity (Fig. 2B) (Pavletich et al. 1993; Cho et al. 1994; Xue et al. 2009).

The Δ40, Δ133, and Δ160 protein isoforms are, respectively, lacking the first 39, 132, and 159 amino acids. The Δ40 isoforms have lost the TA-1 but still retain the TA-2 and the complete DBD. The Δ133 isoforms lack both TA domains and a small part of the first conserved cysteine box of the DBD, whereas the Δ160 isoforms lack both TA domains and the entire first conserved cysteine box of the DBD, retaining the three other conserved cysteine boxes of the DBD.

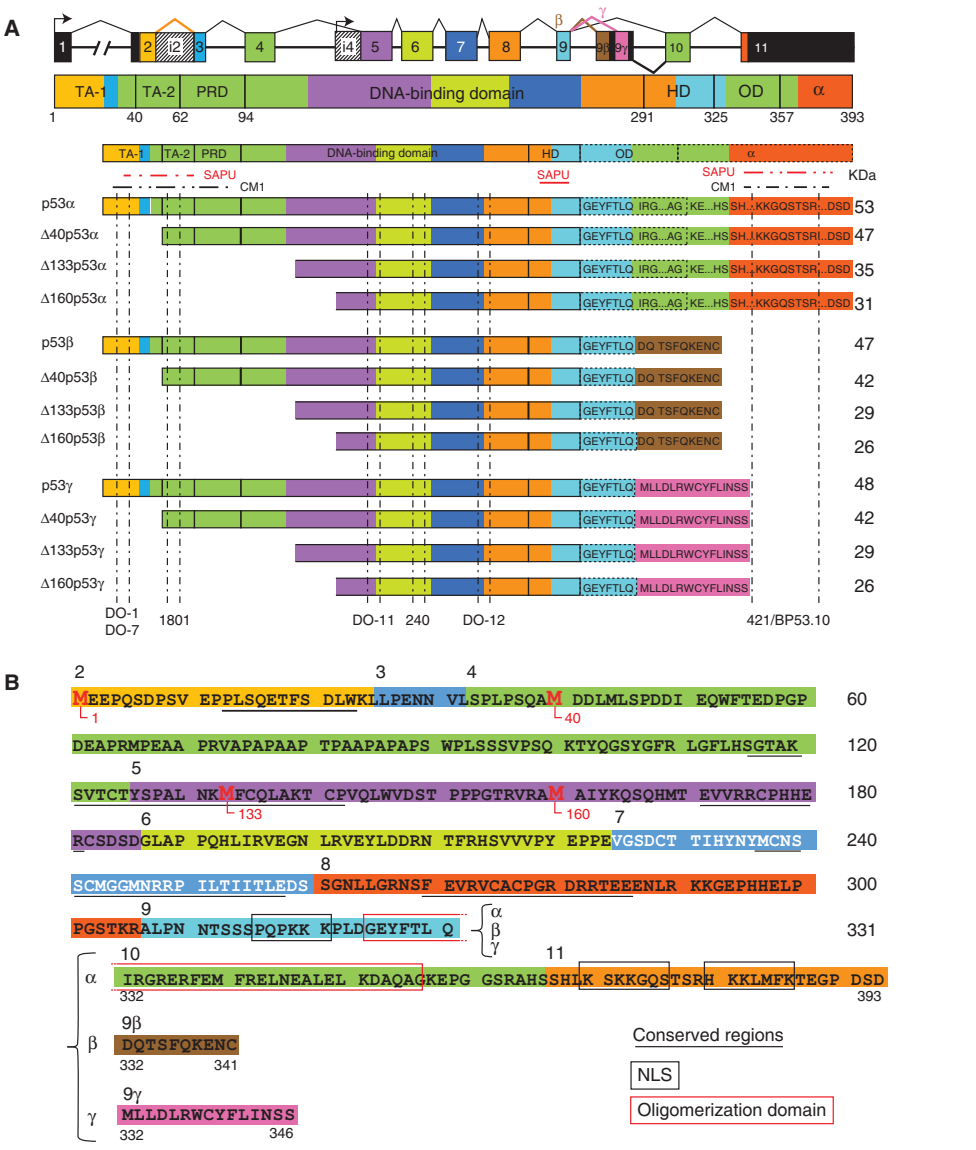

Figure 2. Human p53 protein isoforms. All exons and domains are represented to scale. (*A*) Schematic of the 12 p53 isoforms. The color of the protein domains matches the corresponding exon. p53α is composed of two transactivation domains (TA-1 and TA-2), a proline-rich domain (PRD), the DNA-binding domain (DBD), the hinge domain (HD), the oligomerization domain (OD), and the α regulatory domain. Compared with p53α, Δ40 forms lack the TA-1 because of alternative translation initiation at ATG40. The Δ133 and Δ160 protein isoforms are transcribed from P2 and lack TA-1, TA-2, PRD, and part of the DBD. Regarding the carboxy-terminal isoforms, β and γ forms are attributable to an alternative splicing of intron-9 (brown and pink). As they contain the entire exon-9, they encode the beginning of the OD. The theoretical molecular weight of each protein isoform is indicated. The epitope boundaries of different p53 antibodies are represented by dotted lines. It is important to note that Sapu and CM1 polyclonal antibodies contain multiple epitopes within the first 80 amino acids of p53α and several epitopes in the α-carboxy-terminal regulatory domain, explaining the enhanced detection of p53α compared with the other p53 protein isoforms. (*B*) Amino-acid sequence of human p53 isoforms. The color of the amino-acid sequence matches the encoding exon. The translation initiation methionines (M) are indicated in red. The α, β, or γ peptide sequences are indicated. The underlined amino-acid sequences relate to the conserved cysteine domains in the DBD. Three nuclear localization sequences (NLS) are represented in black boxes. One is common to all isoforms (exon-9) and two are specific of the α forms. The OD encoded by exon-9 and -10 is shown in a red box.

All p53 isoforms, whether transcribed from the P1 or P2 promoter, can alternatively splice exon-9β and -9γ to produce the β- and γ-carboxy-terminal protein domains, replacing part of the oligomerization domain and the entire α domain. The β-carboxy-terminal protein is composed of 10 amino acids, although the γ-carboxy-terminal protein domain is composed of 15 amino acids. Of note, the first seven amino acids of the oligomerization domain are present in the α, β, and γ p53 isoforms.

SCIENTIFIC TOOLS TO INVESTIGATE p53 ISOFORM BIOLOGICAL ACTIVITIES

(RT-q)PCR

The most reliable method to identify and quantify p53 splice variants is (RT-q)PCR (quantitative reverse transcription polymerase chain reaction). Several protocols and a set of primers have been designed and published to detect and quantify p53 isoform mRNAs in cell lines and tumor samples (Khoury et al. 2013). However, there is not always a strict correlation between the expression levels of p53 mRNA variants and p53 protein isoforms because p53 protein isoforms are also regulated at the posttranscriptional level.

Small Interfering RNAs (siRNAs)

We and others have developed several siRNAs and shRNAs targeting specifically distinct exons or introns of human *TP53*, which enable knockdown differentially and specifically subsets of human p53 splice variants (Fig. 1B; Table 1). The siRNAs are essential tools to determine the biological activities of endogenous p53 pro-

Table 1. p53 isoform-specific small interfering RNAs (siRNAs)

	Position on sequence (nt) GenBank accession number (NG_017013)	Exon location	Exon sequence	Targeted forms
si E2	15968–15986	Exon 2	GCAGUCAGAUCCUAGCGUC	p53α, p53β, p53γ, Δ40p53α, Δ40p53β, Δ40p53γ
si 133a	17212–17230	Intron 4	GGAGGUGCUUACACAUGUU	Δ133p53α, Δ133p53β, Δ133p53γ, Δ160p53α, Δ160p53β, Δ160p53γ
si 133b	17271–17289	Intron 4	CUUGUGCCCUGACUUUCAA	Δ133p53α, Δ133p53β, Δ133p53γ, Δ160p53α, Δ160p53β, Δ160p53γ
si E7	18322–18340	Exon 7	GCAUGAACCGGAGGCCCAU	All
si E7/E8	18363–18370/ 18714–18724	Exon 7 and 8	GACTCCAGTGGTAATCTAC	All
si β	19211–19229	Exon 9β	GGACCAGACCAGCUUUCAA	p53β, Δ40p53β, Δ133p53β, Δ160p53β
si γ	19009–19016/ 19285–19295	Exon 9 and 9γ	CCCUUCAGAUGCUACUUGA	p53γ, Δ40p53γ, Δ133p53γ, Δ160p53γ
si Splice	19284–19302	Exon 9β and 9γ	GAUGCUACUUGACUUACGA	p53β, Δ40p53β, Δ133p53β, Δ160p53β, p53γ, Δ40p53γ, Δ 133p53γ, Δ160p53γ
si α	21846–21864	Exon 10	GUGAGCGCUUCGAGAUGUU	p53α, Δ40p53α, Δ133p53α, Δ160p53α

tein isoforms (Bourdon et al. 2005; Fujita et al. 2009; Aoubala et al. 2011; Terrier et al. 2011, 2012, 2013; Marcel et al. 2012, 2014; Wei et al. 2012; Bernard et al. 2013; Mondal et al. 2013).

Antibodies

Several mouse monoclonal antibodies are available to detect endogenous p53 protein isoforms by immunofluorescence, immunohistochemistry (IHC) on paraffin-embedded tissue, and western blotting (Marcel et al. 2013). The epitope of each p53 antibody has been determined by ELISA on an epitope-mapping peptide library (Figs. 2A, 3B; Table 2). The mouse monoclonal antibodies DO-1 and DO-7 recognize the same epitope within the TA-1 domain and can only detect p53α, p53β, and p53γ. The mouse monoclonal antibody 1801 recognizes an epitope within the TA-2 domain and, thus, detects p53α, p53β, p53γ, Δ40p53α, Δ40p53β, and Δ40p53γ. In some cell lines and tissue, 1801 cross-reacts with a protein not related to p53. The mouse monoclonal 421 and BP53.10 recognize a similar epitope within the α domain and, thus, detect p53α, Δ40p53α, Δ133p53α, and Δ160p53α. The other mouse monoclonal antibodies (DO-11, 240, DO-12, and 1620) recognize distinct epitopes within the DBD and should, thus, theoretically detect all p53 protein isoforms. Because p53 protein isoforms can be modified by posttranslational modifications, which alter their migration on SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), it is recommended to transfect cells with siRNA targeting distinct p53 splice variants to validate that the bands detected are p53 protein isoforms (Marcel et al. 2013).

Table 2. p53 isoform-specific antibodies

	Epitope			
	Amino acids	Exon	Sequence	Recognized forms
DO-1	20–25	2	SDLWKL	p53α, p53β, p53γ
DO-7	20–25	2	SDLWKL	p53α, p53β, p53γ
1801	46–55	4	SPDDIEQWFT	p53α, p53β, p53γ, Δ40p53α, Δ40p53β, Δ40p53γ
1620	145–157 + 201–212	5/6	LWVDST PPPGTRV + LRVEYLDDRN TF	ND
DO-11	181–190	5/6	RCSDSDGLAP	All
240	211–220	6	TFRHSVVVPY	All
DO-12	256–267	7/8	TLEDSSGNLLGR	All
SAPU	11–65/ 290–301/ 363–393	2/3/4/ 8/9/ 10/11	EPPLSQ-DPGPDEAPR/RKKGEPHHELPP/ RAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD	All
CM1	1–90/ 379–393	2/3/ 4/11	MEEPQSDPSV.....TPAAPAPAPS/ RHKKLMFKTEGPDSD	p53α, p53β, p53γ, Δ40p53α, Δ40p53β, Δ40p53γ, Δ133p53α, Δ160p53α
421	372–379	11	KKGQSTSR	p53α, Δ40p53α, Δ133p53α, Δ160p53α
BP53.10	374–378	11	GQSTS	p53α, Δ40p53α, Δ133p53α, Δ160p53α

ND, not determined.

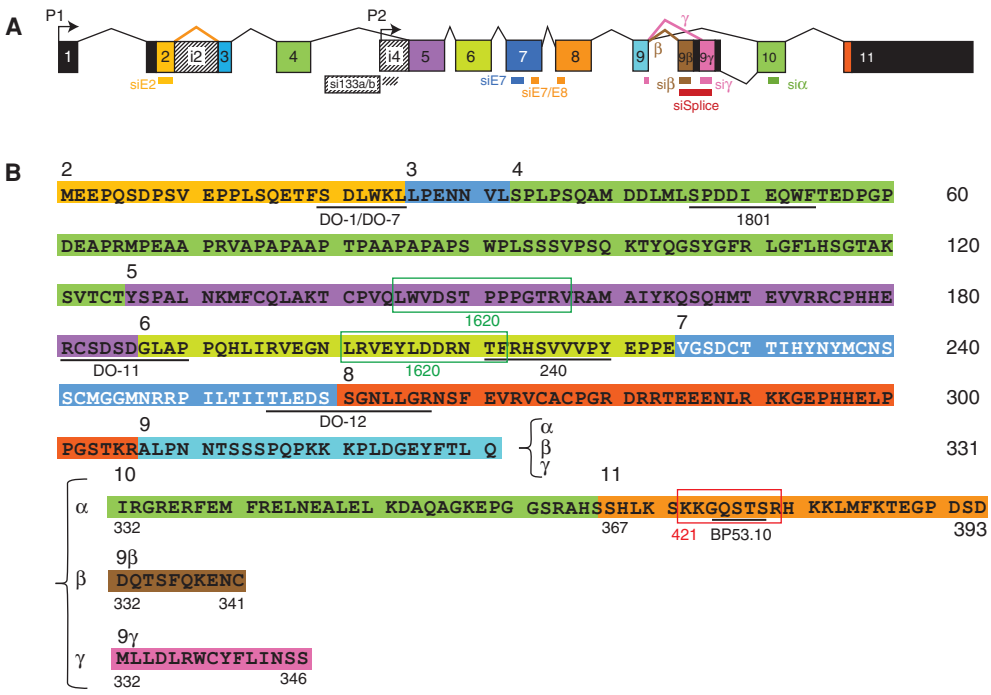

Figure 3. Localization of the human p53 small interfering RNA (siRNA) and p53 antibodies's epitopes. (*A*) p53 isoform-specific siRNAs are represented on the human *TP53* gene. (*B*) p53 antibodies' epitopes on human p53 protein. The underlined amino-acid sequences correspond to the epitopes of the named antibodies (mAb1801, mAb240, mAb DO-11, and mAb DO-12). mAb DO-1 and DO-7 have the same epitope. mAb1620 antibody has a conformational epitope that is represented by green boxes. The mAb 421 epitope is shown in a red box, whereas the mAb BP53.10 epitope is underlined.

Furthermore, posttranslational modifications within epitopes can prevent binding of p53 antibodies. Detection by DO-1 is impaired by phosphorylation of serine-20 (Craig et al. 1999). Similarly, it is well established that binding of 421 antibody is prevented by posttranslational modification of lysine-372, serine-376/378, threonine-377, and histidine-380 (Pospísilová et al. 2000). The antibody BP53-10 could be used to circumvent this problem as it is less sensitive to posttranslational modifications surrounding its epitope.

The rabbit (CM1) and sheep (SAPU) polyclonal antibodies have been raised against bacterially produced recombinant human p53α. They recognize several distinct epitopes within the amino-terminal and α domains so that the detection of p53α is strongly enhanced compared with the other p53 isoforms, thus preventing any expression-level comparisons between p53α and the other isoforms. CM1 and SAPU antibodies have a lower affinity for the Δ133/Δ160 and β/γ forms that lack the amino terminus or carboxyl-terminus epitopes, respectively. Only SAPU recognizes an epitope within the HD, which is common to all p53 protein isoforms. It can, thus, detect Δ133p53β/γ and Δ160p53β/γ, whereas CM1 cannot.

THE p53 ISOFORM RELEVANCE IN CLINIC

In recent years, numerous studies have shown that p53 isoforms are abnormally expressed in breast cancer, melanoma, renal cell carcinoma (RCC), acute myeloid leukemia (AML), colon carcinoma, head and neck tumors (HNSSCs), hepatic cholangiocarcinoma, ovarian cancer, lung tumor, and glioblastoma (Bourdon et al. 2005, 2011; Anensen et al. 2006; Boldrup et al. 2007; Avery-Kiejda et al. 2008; Marabese et al.

2008; Fujita et al. 2009; Song et al. 2009; Hofstetter et al. 2010; Nutthasirikul et al. 2013; Takahashi et al. 2013).

Several studies investigated whether p53 isoforms were associated with cancer patient prognosis. First, it was reported that the evolution from colorectal adenoma to carcinoma was potentially driven by an imbalance of p53β/Δ133p53α ratio in favor of the latter (Fujita et al. 2009). In AML, distinct p53 isoform biosignatures correlate with clinical outcome (Ånensen et al. 2012), whereas in cholangiocarcinoma, down-regulation of the TAp53 isoforms (p53α/p53β/p53γ) combined with up-regulation of the Δ133 forms is associated with a shortened overall survival (Nutthasirikul et al. 2013).

Importantly, several studies in different human cancers have reported that the prognostic value of *TP53* mutation status is improved when combined with p53 isoform expression. Wild-type (WT) *TP53* mucinous or serous ovarian cancer patients expressing Δ40p53α have a better clinical outcome than WT *TP53* mucinous or serous ovarian cancer patients not expressing Δ40p53α. Reciprocally, mutant *TP53* serous ovarian cancer patients expressing Δ133p53α have a better disease-free and overall survival than mutant *TP53* serous ovarian cancer patients not expressing Δ133p53α (Hofstetter et al. 2011, 2012; Chambers and Martinez 2012). Furthermore, in WT *TP53* ovarian cancers, p53β expression is associated with higher risk of recurrence because it is a marker of serous and poorly differentiated cancer (Hofstetter et al. 2010).

In breast cancer, it was reported that p53β expression is associated with smaller tumors and longer disease-free survival in mutant *TP53* tumors, whereas Δ40p53 isoforms expression was found to be associated with the aggressive triple negative subtype (Avery-Kiejda et al. 2014). In another study, it was shown that mutant *TP53* breast cancer patients expressing p53γ have as a good prognosis as WT *TP53* breast cancer patients, whereas mutant *TP53* breast cancer patients devoid of p53γ expression have a particularly poor prognosis (Bourdon et al. 2011).

Altogether, it suggests that the prognostic values of p53β, p53γ, Δ40p53α, or Δ133p53α depend on the *TP53* mutations status and the cancer type.

Other studies have investigated whether p53 isoforms play active roles in cancer formation and treatment. Silent *TP53* mutation or mutations in noncoding regions, such as IRES sequences, introns, or splicing sites, are associated with cancer formation probably because they lead to unbalanced p53 isoform expression despite expressing WT p53 proteins (Hofstetter et al. 2010; Grover et al. 2011; Khan et al. 2013). Furthermore, the pathogenic bacteria *Helicobacter pylori* has recently been shown to induce expression of Δ133 and Δ160 isoforms in gastric epithelial cells, increasing their survival and probably promoting cancer formation (Wei et al. 2012). Moreover, p53 isoforms are involved in response to chemotherapy in vivo in AML and melanoma (Anensen et al. 2006; Avery-Kiejda et al. 2008; Ånensen et al. 2012).

In conclusion, p53 isoforms expression is associated to clinical outcome of cancer patients. For a given cancer type, the accuracy of patient prognosis is greatly improved by combining p53 isoforms expression and *TP53* mutation status, which may be used to predict response to treatment for some cancer types.

ANIMAL MODELS: IN SEARCH OF THE PHYSIOLOGICAL RELEVANCE OF ISOFORMS

The structure of the *TP53* gene is highly conserved through evolution (Bourdon et al. 2005; Chen et al. 2005). Several p53 isoform animal models were, thus, generated to investigate their biological relevance and roles in carcinogenesis.

Zebrafish

The zebrafish *TP53* gene (*Zp53*) has a dual gene structure as its human counterpart (Storer and Zon 2010). In addition to the Zp53α protein, corresponding to the human p53α (Cheng et al. 1997), the gene also encodes Zp53β, ZΔNp53α, and ZΔ113p53α. In addition, in some zebrafish strains, the *Zp53* gene expresses from the inter-

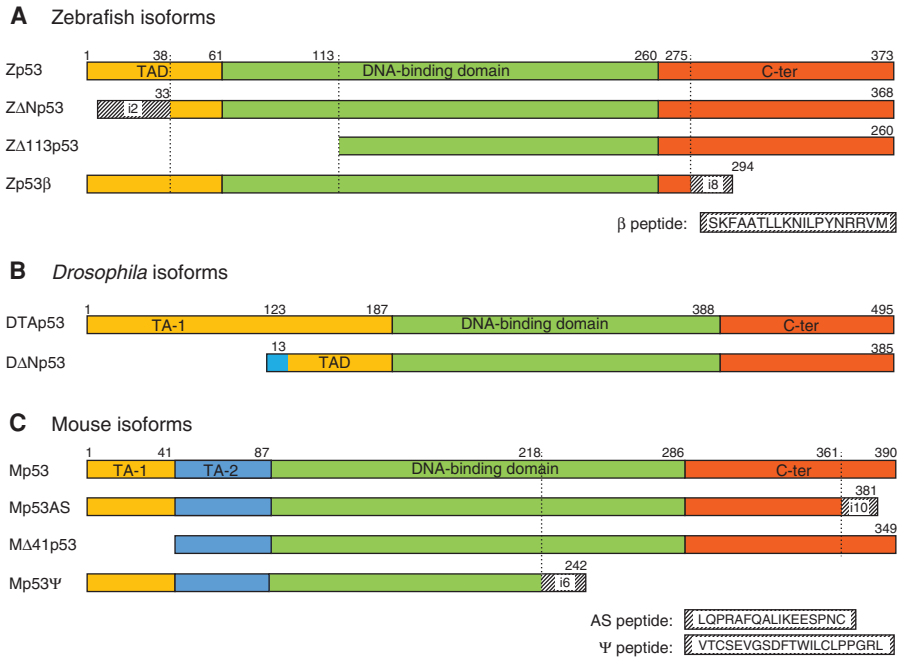

Figure 4. p53 isoforms in animal models. Like human p53 protein isoforms, zebrafish, *Drosophila*, and mouse p53 proteins are also composed of several functional domains: the transactivation domain (TAD, yellow), the DNA-binding domain (DBD, green) and the carboxy-terminal regulatory domain containing the oligomerization domain (C-ter, orange). For each species, all domains are represented to scale compared with the full-length protein of the corresponding species. Hatched protein domains are encoded by cryptic exons within introns (the corresponding intron is indicated). (*A*) Zebrafish p53 isoforms. In the ZΔNp53 isoform, the first 33 amino acids are encoded by a cryptic exon in intron-2. In Zp53β, the last 19 amino acids are encoded by a cryptic exon in intron-8. Of note, Zp53β is not homologous to human p53β. (*B*) *Drosophila* p53 protein isoforms. Like in the human *TP53* gene family, DΔNp53 (also named Dp53) protein is encoded by an mRNA transcribed from an internal promoter. DΔNp53 is homologous to Δ40p53/Δ133p53/ΔNp63/ΔNp73 proteins. Only DTAp53 protein contains the conserved box I (FxxLW) corresponding to the transactivation domain (TA-1) of the p53 protein family. (*C*) Mouse p53 isoforms. In Mp53AS protein (alternatively spliced [AS]), the last 26 amino acids of Mp53α are substituted by 17 others encoded by a cryptic exon in intron-10. Mp53AS protein is homologous to human p53β. MΔ41p53 produced by alternative splicing of intron-2 is homologous to human Δ40p53α. The Mp53Ψ is produced by alternative splicing of intron-6 adding 21 new amino acids as indicated. Up to now, no homologous protein to Mp53Ψ has been found in other organisms or in human cells expressing the WT *TP53* gene.

nal promoter TA2Zp53, TA3Zp53, TA4Zp53, and TA5Zp53 because of a polymorphism (Fig. 4A).

ZΔNp53α is produced through retention of intron-2, similarly to the human Δ40p53 forms. However, ZΔNp53α is not completely identical to the human form as the translation is initiated in intron-2 and substitutes the 38 first amino acids of canonical p53 by 33 different amino acids constituting a different TA domain (Davidson et al. 2010). ZΔNp53α accumulates in response to γ-ray and is able to form a protein complex with Zp53α through the OD. When coexpressed with Zp53α, overexpression of ZΔNp53α induces developmental malformations (hypoplasia, head malformation, somites, and eyes). This phenotype does not appear after overexpression of ZΔNp53α mutated in the oligomerization domain or in Zp53 morpholino-depleted embryo, suggesting that ZΔNp53α acts through oligomerization with Zp53 isoforms during development.

ZΔ113p53α is homologous to human Δ133p53α. It is produced from the internal promoter, which is transactivated by Zp53α (Chen et al. 2005; Marcel et al. 2010b). ZΔ113p53α oligomerizes with Zp53α inhibiting apoptosis by differentially modulating Zp53 target gene expression (Ou et al. 2014). ZΔ113p53α induces expression of cell-cycle arrest genes, such as p21 and cyclin-G_1 and of antiapoptotic genes, such as Bcl-XL, while inhibiting expression of proapoptotic genes like *Reprimo* or Bax (Chen et al. 2009). Furthermore, ZΔ113p53α is strongly induced after DNA double-strand break (DSB) and activates the DNA–DSB repair pathways, notably by up-regulating the transcription of repair genes such as RAD51, RAD52, or lig4 (Gong et al. 2015).

Recently, Shi et al. reported that a natural polymorphism, a 4-bp deletion within intron-4, creates four new upstream translation initiation sites in frame to the ZΔ113p53 open reading frame. This leads to four protein isoforms with a putative TA domain named TA2p53, TA3p53, TA4p53, and TA5p53. They lack the 93 first amino acids of Zp53α that are, respectively, replaced by 65, 45, 18, and 9 amino acids (Shi et al. 2015). These isoforms coexpressed with Zp53α confer resistance to irradiation. In the same publication, Shi et al. also reported the alternative splicing of *Zp53* intron-8 leading to Zp53β. However, Zp53β is not homologous to human p53β.

Drosophila

The *Drosophila melanogaster TP53* gene (*Dmp53)* is the single *Drosophila* ortholog of the mammalian p53 family and has a dual gene structure with an internal promoter. It encodes proteins homologous to p53, p63, and p73 (Lu et al. 2009), and it is thought that, as the unique member of the p53 family, it is likely that *Dmp53* carries the ancestral functions of all p53 family genes. *Dmp53* transcribes four different mRNA variants but, so far, only two p53 protein isoforms could be detected: DTAp53 and DΔNp53 (Fig. 4B). DTAp53 contains, in the first 40 amino acids, the highly conserved TA domain present in mammalian p53α (Bourdon et al. 2005). DΔNp53 (also called Dp53), expressed from the internal promoter, lacks the 123 first amino acids of DTAp53, which are replaced by 13 different ones encoded by a cryptic exon. DΔNp53 does not contain the conserved TA domain but is still able to transactivate. DΔNp53 was the first *Drosophila* p53 isoform cloned and was thus called Dp53 as the investigators thought they had cloned full-length *Drosophila* p53 (Brodsky et al. 2000; Jin et al. 2000; Ollmann et al. 2000). However, DΔNp53 is homologous to the amino-terminally truncated isoforms of the p53 family proteins (Δ40p53, ΔNp63, or ΔNp73). The literature on *Drosophila* p53 is, thus, confusing as most genetic studies do not discriminate between DTAp53 and DΔNp53 isoforms, reporting phenotype associated with *Dmp53* gene as being mediated by Dp53 protein.

Another source of confusion is that mutant p53 flies referred both to p53-null flies and to transgenic flies overexpressing single-point missense mutant of Dmp53 (UAS-Dmp53R155H or UAS-Dmp53H159N). However, p53-null mutation leads to complete loss of p53 protein expression and, thus, p53 activity, whereas missense mutation leads to overexpression of mutant p53 proteins that are biochemically and biologically very active as they directly interact with numerous proteins modifying, thus, gene expression and cell responses. p53-null mutation can lead to different phenotypes than missense p53 mutation as shown in *Drosophila* and mammalian models (Brodsky et al. 2000; Ollmann et al. 2000; Jassim et al. 2003; Lang et al. 2004; Olive et al. 2004; Wells et al. 2006; de la Cova et al. 2014; Simón et al. 2014)

The *Dmp53* gene is involved in apoptosis through the Reaper–Hid–Grim cascade by directly regulating Reaper expression in response to damage (Brodsky et al. 2000, 2004). However, its physiological activity is not limited to apoptotic pathways. Recent studies unravel the primordial roles of *Drosophila* p53 protein isoforms in tissue regeneration by directly controlling apoptosis-induced proliferation (AiP), compensatory proliferation (CP), cell differentiation, organ size, system growth, and cell competition (Peterson et al. 2002; Jassim et al. 2003; Wells et al. 2006; Stieper et al. 2008; Mendes et al.

2009; Fan et al. 2010; Mesquita et al. 2010; Morata et al. 2011; Wells and Johnston 2012; de la Cova et al. 2014; Simón et al. 2014). This is consistent with *Dmp53* being involved in life-span control (Bauer et al. 2005, 2010; Waskar et al. 2009; for review, see Martín et al. 2009; Kashio et al. 2014; Mollereau and Ma 2014).

However, the contribution of each *Drosophila* p53 protein isoform in those biological phenomena is still poorly understood. Recently, Dichtel-Danjoy et al. (2013) investigated the respective roles of DTAp53 and DΔNp53 overexpression in apoptosis and AiP in the developing wing imaginal disc. AiP is a phenomenon in which cells undergoing apoptosis after a stress event (i.e., "undead cells") secrete mitogens, such as Wingless (Wg), Decapentaplegic (Dpp), or Hedgehog (Hh) (homologous to human Wnt, TGF-β, or Hedgehog, respectively) to promote proliferation of neighboring cells to restore the normal organ and body size. Thus, at the same time that the stress event eliminates a large number of cells, it also generates a stimulus to proliferation. AiP is directly controlled by *Dmp53* (Wells et al. 2006; Dichtel-Danjoy et al. 2013; Simón et al. 2014). Both DTAp53 and DΔNp53 protein isoforms were capable of activating apoptosis but they used different molecular mechanisms. However, only DΔNp53 (not DTAp53) can induce Wg expression in "undead" cells and enhanced proliferation of neighboring cells in Dmp53-null flies, suggesting that DΔNp53 is the main isoform that regulates AiP. DTAp53 and DΔNp53 seem, thus, to have distinct biological functions.

Mouse

The mouse *TP53* gene (*MsTP53*) has a dual gene structure as its human counterpart. To date, four mouse p53 protein isoforms have been published: Mp53α, Mp53AS, MΔ41p53α, and Mp53Ψ (Fig. 4C). Mp53α is the equivalent to the canonical human p53α. Mp53AS is a carboxy-terminal splicing variant because of retention of part of intron-10. It encodes a 2-kDa shorter protein with different carboxy-terminal amino acids, which has a strong homology with the human β p53 proteins. Mp53AS can bind DNA and modulate p53-target-gene expression in a promoter-dependent manner. Mp53AS can induce apoptosis when expressed in absence of Mp53α (Almog et al. 1997). However, when Mp53AS and Mp53α are coexpressed, cells do not induce apoptosis in response to damage (Wolf et al. 1985; Almog et al. 2000). Mp53AS and Mp53α oligomerize together and bind DNA to regulate gene expression (Wu et al. 1994). Therefore, Mp53AS and Mp53α harbor completely different/opposite activities when they are expressed alone or in combination.

Recently, two knockin p53 mice, $Mp53^{\Delta 24}$ and $Mp53^{\Delta 31}$-expressing carboxy-terminal truncated mutants of p53 have been generated. The $Mp53^{\Delta 24}$ mice contain a stop mutation at codon 367 in the beginning of exon-11 so that the mice express $Mp53^{\Delta 24}$, $M\Delta 41p53^{\Delta 24}$ but also the WT Mp53AS and Mp53Ψ proteins, because alternative splicing of intron-10 can still occur (Hamard et al. 2013). The $Mp53^{\Delta 31}$ mice contain a stop mutation at codon 360 at the end of exon-10 so that the mice express $Mp53^{\Delta 31}$, $M\Delta 41p53^{\Delta 31}$, and WT Mp53Ψ. The $Mp53^{\Delta 31}$ mice do not express Mp53AS (Simeonova et al. 2013). In both studies, the *MsTP53* gene has been extensively sequenced to ensure the absence of any additional mutations in *MsTP53* exons and introns. Interestingly, the $Mp53^{\Delta 24}$ and $Mp53^{\Delta 31}$ mice present different phenotypes despite being of similar mixed genetic background (BL6/129Sv). The $Mp53^{\Delta 24}$ and $Mp53^{\Delta 31}$ mice have different blood cell counts and different sizes of heart, thymus, spleen, testis, and cerebellum. $Mp53^{\Delta 31}$ mice also develop oral leukoplakia (100%) and pulmonary fibrosis (87%), traits not reported for $Mp53^{\Delta 24}$ mice. One of the most intriguing differences is in the repopulating capabilities of hematopoietic stem cells (HSCs) from embryos of the $Mp53^{\Delta 24}$ and $Mp53^{\Delta 31}$ mice. WT matching mixed genetic background mice (BL6/129Sv) were lethally irradiated to destroy the bone marrow cells. The irradiated mice were then transplanted with HSCs from $Mp53^{\Delta 24}$, $Mp53^{\Delta 31}$, or WT *MsTP53* mice (BL6/129Sv). In both laboratories, the transplantation of HSC from WT *MsTP53* mice rescued 100% of the irradiated mice by regenerating the bone marrow, al-

though none of the irradiated and nontransplanted mice survived, indicating that both laboratories master this assay. Importantly, the transplantation of HSC from Mp53$^{\Delta 31}$ mice completely fails to repopulate the bone marrow and to rescue, thus, the irradiated mice (0% rescue, $n = 5$), whereas the transplantation of HSC from Mp53$^{\Delta 24}$ mice rescue ~70% ($n = 6$) of the irradiated mice by repopulating the bone marrow (Hamard et al. 2013; Simeonova et al. 2013).

Knowing the potent transcriptional activities of Mp53AS and its tissue-specific expression, it is likely that the different phenotypes of the Mp53$^{\Delta 24}$ and Mp53$^{\Delta 31}$ mice are attributable to differential expression of Mp53AS. Further investigations will be required to determine the physiological roles of Mp53AS in tissue regeneration in mice.

Recently, an additional isoform attributed to the alternative splicing of intron-6, Mp53Ψ (also named p53Ψ) has been described. This results from the physiological insertion of the last 55 nucleotides of intron-6, which rapidly leads to a stop codon. Mp53Ψ is thus a very short p53 isoform containing the TA and proline domains but no DBD (Senturk et al. 2014). Mp53Ψ may act as a prometastatic factor in promoting epithelial–mesenchymal transition and regulating mitochondrial activity together with cyclophilin-D. Interestingly, Mp53Ψ seems to be expressed after damage in specific stem cells during tissue regeneration in mice. In liver injured with CCL4, the lesions get smaller concomitantly to higher expression of Mp53Ψ. This is a fascinating result as it indicates that the *Msp53* gene is involved in tissue regeneration in mammalians. Furthermore, it opens new perspectives as p53 isoform expression could be manipulated to control organ regeneration in mammalians.

Mp53Ψ mRNA expression is unequivocal in regenerating lung or liver mouse tissues; further investigations will be required to confirm physiological p53Ψ expression in WT human cells. So far, endogenous human p53Ψ protein could only be detected in one cancer cell line (Hop62) that bears a mutation in the acceptor-splicing site of human *TP53* intron-6. Such a mutation abolishes the canonical splicing of intron-6 and promotes the insertion of the last 49 nucleotides of human *TP53* intron-6 leading to a p53Ψ-like protein as shown by the investigators (Senturk et al. 2014). The development of human/mouse p53Ψ-specific antibodies and siRNA would greatly help to assess p53Ψ expression and study its biological activities.

The MΔ41p53α isoform has also been genetically investigated. MΔ41p53α (also named p44) is the mouse counterpart of human Δ40p53α. MΔ41p53α overexpression in WT *Mp53* mice leads to a smaller animal with premature aging and shorter life span. These effects have been attributed to abnormal insulin-like growth factor (IGF) signaling, driving a reduced cellular proliferation with increased senescence (Maier et al. 2004; Gambino et al. 2013), inhibition of embryonic stem cell (ESC) differentiation (Ungewitter and Scrable 2010), neurodegeneration (Pehar et al. 2010, 2014), and impaired β-cell proliferation (Hinault et al. 2011), which suggests that the MΔ41p53 transgenic mice have altered tissue-regeneration capabilities.

To genetically investigate the biological activities of Δ133p53 isoforms, Slatter et al. (2011) have generated knockin mutant *TP53* mice that are deleted of exon-3 and -4 (*Ms*Δ*122p53*), expressing ubiquitously Δ133p53-like protein (Δ122p53α) and probably Δ122p53AS in a tissue-dependent manner because of alternative splicing of intron-10. The homozygote *Ms*Δ*122p53* mice show enhanced proinflammatory phenotype, features of autoimmune disease, and early tumor onset (Campbell et al. 2012; Sawhney et al. 2015). It was thus thought that Δ122p53 proteins were oncogenic. However, it was recently genetically shown that Δ122p53 proteins enhance the tumor-suppressor activities of an attenuated p53 mutant deleted of the proline domain (MΔpro) (Slatter et al. 2015).

Altogether, the different animal models are consistent in demonstrating that the p53 isoforms are potent and essential components of the p53 pathway, being involved in cancer, aging, tissue regeneration, glucose metabolism, embryo development, immune system, and bacterial infection (Wei et al. 2012). The balance

between the different p53 isoforms is crucial in determining cell-fate outcome.

REGULATION OF HUMAN p53 ISOFORM EXPRESSION/ACTIVITIES

p53 isoform expression is regulated at the transcriptional level by modulating the *TP53* promoter activities and the alternative splicing of intron-2 and intron-9.

In addition to the epigenetic events that regulate the tissue-specific activity of the p53 promoters, the internal *TP53* promoter activity is also influenced by several polymorphisms, particularly the 16-bp insertion in intron-3 (pin3) and the R72P polymorphism in exon-4 altering thus Δ133p53 isoform expression (Fig. 1A) (Bellini et al. 2010; Marcel et al. 2012). In addition, the internal *TP53* promoter is transactivated by p53α, diverse p63/p73 isoforms, and p68 (Moore et al. 2010; Aoubala et al. 2011; Marcel et al. 2012).

SRSF1 and SRSF3, a highly conserved family of splicing factors frequently deregulated in cancer, regulate the alternative splicing of *TP53* intron-9 inhibiting retention of exon-9β/9γ (Tang et al. 2013; Marcel et al. 2014). Interestingly, TG003, a small drug inhibitor of the Cdc2-like kinases (Clks) that activate SRSF1 and SRSF3, increases endogenous p53β/γ expression inhibiting cell proliferation in a p53 isoform-dependent manner (Marcel et al. 2014).

The alternative splicing of *TP53* intron-2 leads to Δ40p53. Its expression is influenced by a G-quadruplex structure within intron-3, which is strengthened by the pin3 polymorphism that contains further G-quadruplex structures (Marcel et al. 2011).

p53 isoform expression is also regulated at the translational and posttranslational levels. The Δ40p53 isoforms can be translated from IRES controlled by IRES transactivating factors (ITAFs), such as polypyrimidine-tract-binding protein (PTB), dyskerin, DAP5, annexin A2, and PTB-associated splicing factor (PSF) (Grover et al. 2008; Sharathchandra et al. 2012; Weingarten-Gabbay et al. 2014).

Camus et al. (2012) have reported that all p53 isoforms are ubiquitinated and degraded by the proteasome. However, although MDM-2 binds to all p53 protein isoforms, it can only promote the ubiquitination and degradation of p53β. MDM2 binds probably Δ133p53 isoforms (α, β, or γ) through the DBD (Wallace et al. 2006) and/or the α-carboxy-terminal domain (Poyurovsky et al. 2010) because the Δ133p53 isoforms have lost the MDM2-binding domain present in the amino-terminal end of p53α, p53β, and p53γ.

In addition to proteasome degradation, Δ133p53α is also degraded by autophagy during replicative senescence. Pharmacological inhibition of autophagy by bafilomycin A1 restores Δ133p53α expression levels. The autophagic degradation of Δ133p53α induces senescence and is inhibited by direct interaction of the chaperone-associated E3 ubiquitin ligase STUB1/CHIP with Δ133p53α (Horikawa et al. 2014).

Altogether, expression of each p53 isoform is tightly and differentially regulated, enabling dynamic and accurate triggering of the appropriate cellular response to damage. The diverse regulations of p53 isoforms expression could be used as therapeutic targets to trigger controlled cell-fate outcome.

p53 ISOFORM BIOLOGICAL ACTIVITIES

p53α, p53β (Mp53AS), p53γ, Δ40p53α (MΔ41p53α), and Δ133p53α (ZΔ113p53α and MΔ122p53) have been shown to differentially regulate gene expression and to be biochemically and biologically active either alone or in combination. Δ133p53β has recently been shown to regulate cell stemness; however, its molecular mechanism is still unknown (Arsic et al. 2015).

Over the past 10 years, using diverse human cell lines and animal models, all data have consistently indicated that the balance of expression among the p53 isoforms define the p53-mediated cell response to trigger after damage, virus/bacterial infection, or cell signals (Maier et al. 2004; Chen et al. 2009; Fujita et al. 2009; Medrano et al. 2009; Davidson et al. 2010; Pehar et al. 2010, 2014; Ungewitter and Scrable 2010; Aoubala et al. 2011; Hinault et al. 2011; Slatter

et al. 2011, 2015; Terrier et al. 2012; Wei et al. 2012; Bernard et al. 2013; Dichtel-Danjoy et al. 2013; Gambino et al. 2013; Mondal et al. 2013; Silden et al. 2013; Horikawa et al. 2014; Marcel et al. 2014; Takahashi et al. 2014; Arsic et al. 2015; Gong et al. 2015). The p53 isoforms thus play primordial roles in cell-cycle progression, programmed cell death, senescence, inflammation, stem-cell renewal and differentiation, aging, neurodegeneration, glucose metabolism, angiogenesis, embryo development, and cancer.

The manipulation of p53 isoforms using splicing-factor inhibitors, autophagy inhibitors, DNA damage, p53 isoform overexpression, and/or siRNA targeting, specifically the p53 isoforms, enables the triggering of different cell-fate outcomes in response to the same damage (Aoubala et al. 2011; Horikawa et al. 2014; Marcel et al. 2014; Gong et al. 2015). Mechanistically, p53 isoforms oligomerize so that, for example, the oligomers composed of p53β and p53α regulate different p53-responsive genes than the ones composed of p53γ and p53α or Δ133p53α and p53α (Fujita et al. 2009; Aouba et al. 2011; Bernard et al. 2013; Marcel et al. 2014; Solomon et al. 2014). p53 isoform expression is cell-type-specific and several p53 isoforms are always concomitantly coexpressed or are mutually exclusive (Fig. 5). Hence, the p53-mediated cell response would be the sum of the activities of each oligomer of the coexpressed p53 isoforms in a given tissue. It implies that none of the p53 isoforms, including canonical p53α, is able to abolish the activity or expression of the other coexpressed p53 isoforms. It is thus impossible to define a p53 isoform as oncogene or tumor suppressor because its activity depends on the cell context. Hence, although p53β in T cells or normal human fibroblasts prevents cell growth and induces senescence (Fujita et al. 2009; Mondal et al. 2013), Marcel et al. (2014) showed that endogenous p53β/γ in MCF-7 cells inhibit cell proliferation by promoting G_1-cell-cycle arrest and cell death under standard culture conditions, but promote cell proliferation after treatment with TG003. In the Δ133p53-like mouse model (*MsΔ122p53*), although Δ133p53α/Δ122p53 inhibits WT p53 tumor suppressor activity, it has recently been reported that Δ122p53 proteins enhance the tumor-suppressor activities of an attenuated p53 mutant deleted of the proline domain (MΔpro) (Slatter et al. 2015). This is consistent with clinical studies. Mutant *TP53* serous ovarian cancer patients expressing Δ133p53α have better disease-free and overall survival than mutant *TP53* serous ovarian cancer patients not expressing Δ133p53α (Hofstetter et al. 2011, 2012; Chambers and Martinez 2012).

CONCLUSION AND INSIGHTS

Based on the data gathered over the past 10 years by several laboratories using diverse human cell lines and animal models, we can now assert that p53 isoforms are physiologically active and potent proteins. However, their activities are cell-type-dependent. The misregulation of p53 isoform expression leads to cancer, premature aging, (neuro)degenerative diseases, inflammation, and embryo malformations. The data leads us then to realize that a p53-mediated cell response is not driven by a single protein, canonical p53, but is in fact the sum of the activities of the coexpressed p53 isoforms in a given tissue. Furthermore, a p53-mediated cell response is broader than just preventing cancer formation by controlling cell proliferation and cell death. In fact, the effects of p53 isoform expression misregulation are consistent with a physiological role of p53 isoforms in organ maintenance/regeneration as observed in *Drosophila*, zebrafish, and mouse p53 isoform animal models (Jassim et al. 2003; Chen et al. 2005, 2009; Medrano et al. 2009; Davidson et al. 2010; Dichtel-Danjoy et al. 2013; Hamard et al. 2013; Simeonova et al. 2013; De la Cova et al. 2014; Kashio et al. 2014; Senturk et al. 2014; Simón et al. 2014; Gong et al. 2015). The balance between the different p53 isoform is crucial in predicting cell-fate outcome. Therefore, deciphering the p53 isoforms combinatorics (p53 isoforms' code) offers fascinating and important new perspectives to treat cancer, degenerative diseases and aging.

Cracking the p53 isoforms' code may seem a daunting task because of the high number of p53 isoforms and posttranslational modifications.

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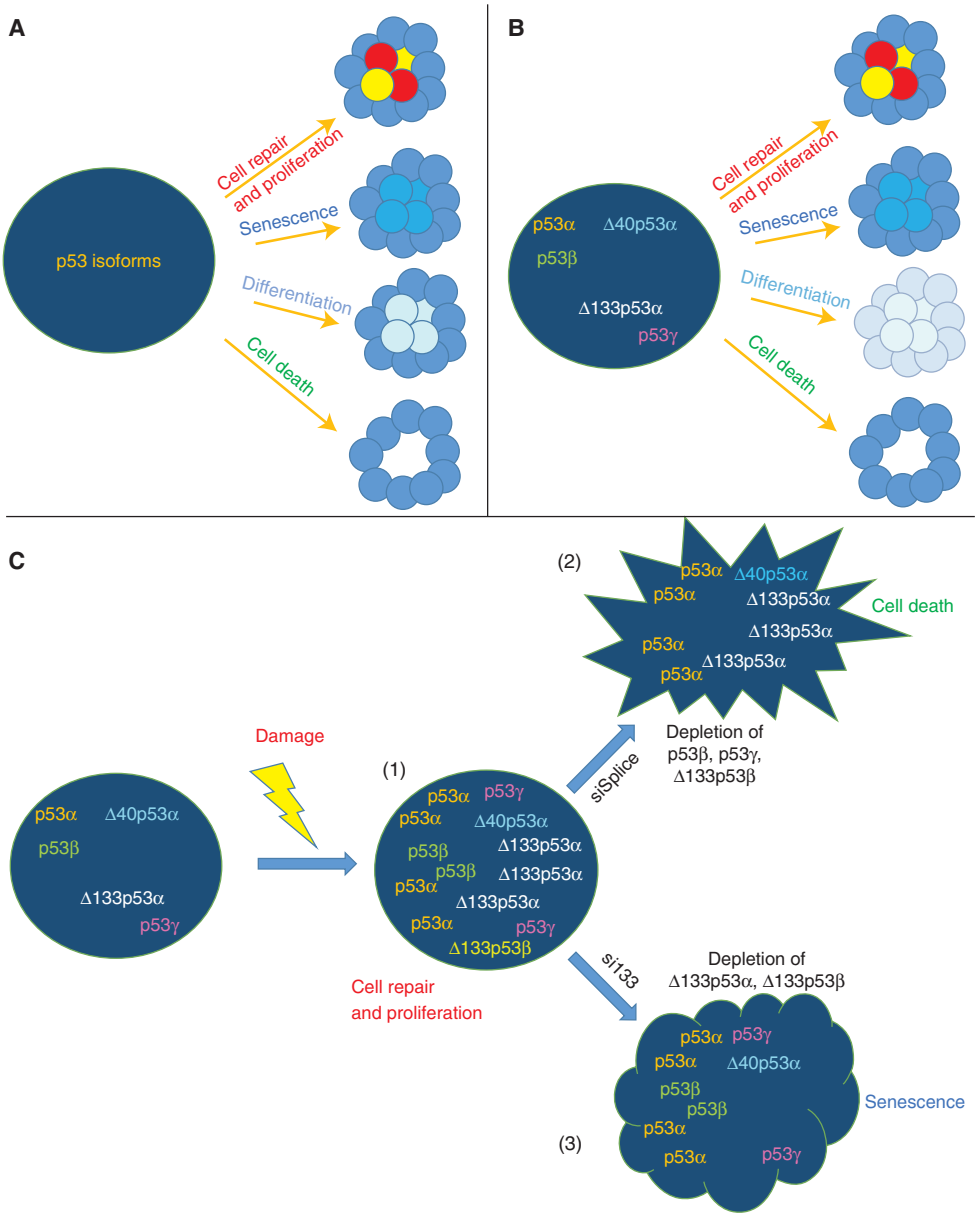

Figure 5. Biological model. (*A*) p53 isoforms define cell-fate outcomes in response to intracellular and extracellular cell signals—cell survival/proliferation, senescence, differentiation, or programmed cell death. (*B*) The cell type (epigenetic) defines p53 isoform expression and the possibilities of cell-fate outcomes. (*C*) In response to damage, p53 isoforms are differentially regulated and define cell-fate outcome. (1) In this theoretical cell, the coexpressed p53 isoforms orchestrate cell repair and allow cell proliferation in response to damage. However, it is important to note that the cell response to the same damage can be drastically changed by manipulating expression of only a subset of p53 isoforms using p53 isoform-specific small interfering RNA (siRNA). (2) Hence, after depletion of p53β, p53γ, and Δ133p53β using the siRNA siSplice, the cell response to the same damage is to induce cell death, (3) although, after depletion of Δ133p53α and Δ133p53β using the siRNA si133, the cell response to the same damage is to induce senescence. (This model is based on data in Wu et al. 1994, Almog et al. 2000, Yin et al. 2002, Chen et al. 2009, Fujita et al. 2009, Ungewitter and Scrable 2010, Aoubala et al. 2011, Terrier et al. 2012, Marcel et al. 2014, Slatter et al. 2015, and Gong et al. 2015.)

However, the p53 isoforms are rarely coexpressed all together at once; some isoforms are mutually exclusive, whereas others are always coexpressed so that the number of p53 isoform combinations is limited. Cracking the p53 isoforms' code seems thus an achievable and necessary task to accurately predict response to cancer treatment and to control stem-cell renewal and differentiation in the treatment of degenerative diseases and aging. It offers new exciting perspectives in regenerative medicine.

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