Classical and novel roles of p53 and prospects for anticancer therapy

José J. Fuster [1], Silvia M. Sanz-González [1], Ute M. Moll [2], and Vicente Andrés [1]

[1] Vascular Biology Unit, Instituto de Biomedicina de Valencia (IBV-CSIC), Spanish Council for Scientific Research, 46010 Valencia, Spain

[2] Department of Pathology, State University of New York at Stony Brook, Stony Brook, NY, USA.

TOTAL WORDS: 4008

KEY WORDS: p53, MDM2, cancer, aging, mitochondria, genetically-engineered mice, anticancer therapy

Send correspondence to:

Vicente Andrés, Instituto de Biomedicina de Valencia, Spanish Council for Scientific Research, Jaime Roig, 11, 46010 Valencia (Spain)

e-mail: vandres@ibv.csic.es.

Abstract

The tumor suppressor p53 is a transcription factor frequently inactivated in human tumors, therefore restoring its function has been considered an attractive approach to restrain cancer. Classically, p53-dependent growth arrest, senescence and apoptosis of tumor cells have been attributed to transcriptional activity of nuclear p53. Notably, wild-type p53 gain-of-function enhances cancer resistance in the mouse but this approach also accelerates aging in some models, possibly due to altered p53 activity. Therefore, the emerging evidence recognizing mitochondrial transcription-independent activities of p53 has raised high expectations. Here, we review new developments in transcription-dependent and transcription-independent p53 functions, as well as recent advances in targeting p53 for cancer treatment and the pitfalls of moving from the laboratory to the clinical setting.

1. Introduction

p53 has been the center of intensive research ever since it was described that most human cancers exhibit inactivating mutations or altered regulation of this protein [1, 2]. p53 mediates the cellular response to a variety of stresses by activating different downstream effectors depending on the type of cell and the nature of the cellular stress. It is widely accepted that p53 exerts its tumor suppression activity by regulating the transcription of several genes involved in cell cycle and apoptosis regulation, among other processes [3]. However, even after more than two decades of research, important questions about transcription-dependent p53 activities remain unanswered. Moreover, novel transcription-independent mechanisms of p53 action have been identified that link p53 to the intrinsic mitochondrial apoptotic pathway (eg. via its interaction with anti- and proapoptotic members of the BCL family of mitochondrial permeability regulators). In the next sections, we review the well-established mechanisms of p53 function as a transcription factor and its regulation and discuss in detail the results from different animal studies. These studies have conclusively demonstrated the key role of p53 in tumor suppression, but have also suggested a potential role of p53 in regulating longevity and thus have shed doubt on the appropriateness of trying to achieve tumor resistance by increasing p53 activity. Additionally, we describe some pharmacological strategies aimed at restoring p53 transcriptional function in tumors and discuss novel transcription-independent mechanisms of p53 proapoptotic activity which might in the future lay the foundations of new therapeutic approaches.

2. p53, the guardian of the genome

The transcription factor p53 plays a key role in preventing DNA damage, for which it has earned the nickname ‘guardian of the genome’. Fig. 1A depicts the main domains of p53 that have been implicated in the regulation of its function. p53 is expressed ubiquitously as an inactive protein that has a very short half-life (20-30 min) and is present at low levels in unstressed cells. However, multiple conditions can lead to a rapid increase of p53's cellular levels and to its activation. These include direct DNA damage, damage to

components involved in the proper handling of the genetic material (such as the mitotic spindle), hypoxia, oncogenic signalling, ribonucleotide depletion and exposure to nitric oxide, among others [4]. Once activated, p53 regulates positively or negatively the transcription of more than 150 genes involved in a variety of cellular processes (Fig. 1B, Table 1 online Supplement).

The response to p53 activation is variable and highly dependent on both the type of cell and the nature of the damaging agent/cellular stress. Active p53 can induce both reversible growth arrest in G1 or G2 phases of the cell cycle, and cellular senescence. This cytostatic action of p53 is important to deter the expansion of tumor-prone cells and to repair DNA damage. In cells beyond repair, p53 can induce apoptosis by stimulating the extrinsic death receptor pathway or, more importantly, the intrinsic mitochondrial pathway.

It is widely accepted that activation of p53 is mainly brought about by protein stabilization and conversion from a latent to an active form. Both processes are regulated by posttranslational changes, including phosphorylation, acetylation, ubiquitination and protein-protein interactions [5, 6]. A key regulator of p53 function is the murine double minute 2 (MDM2) oncoprotein, which inhibits p53 at least through two mechanisms: a) MDM2 binds to the transcriptional activation domain of p53, thus blocking its interaction with the basic transcription machinery [7]; and b) MDM2 promotes the degradation and nuclear export of p53 by acting as a p53-specific E3 ubiquitin ligase [8-11]. Importantly, *MDM2* is a transcriptional target of p53, so that a negative feed-back loop is generated whereby increased p53 activity leads to expression of its main negative regulator [12, 13] (Fig. 1B). This regulatory pathway seems to be important in vivo, since p53 inactivation abrogates the early embryonic lethality caused in mouse by *Mdm2* deficiency [14, 15]. The ability of MDM2 to inhibit p53 is modulated by covalent modifications of both proteins and by other regulatory proteins. For instance, p53 activation by oncogenic signaling depends mainly on the induction of $p19^{Arf}$ (ARF) (Fig. 1B), the product of an alternative transcript of the *INK4A* tumor suppressor gene which also encodes for $p16^{Ink4}$. Using genetically-altered mice, Efeyn et al have very recently shown that p53 tumor suppression activity is abolished in the absence of ARF, thus suggesting that oncogenic signalling is the main event leading to p53-dependent tumor protection [16]. ARF binds to and blocks the E3 ubiquitin ligase activity of MDM2, thereby preventing MDM2-mediated p53 proteolysis [8, 17-19]. Recently, several other p53 E3 ligases were identified, namely PIRH2, COP1, ARF-BP1/Mule and cullin 8 [20-22], whose in vivo importance for p53 regulation remains to be elucidated

3. p53-deficient mice reveal the tumor suppressor function of p53

Mutations in p53 are a hallmark of at least half of all human cancers. p53 was discovered in 1979 as a 53 kDa host protein associated with the oncogenic SV40 large T-antigen in transformed cells [23-25]. The generation and characterization of *p53*-null mice ($p53^{-/-}$) conclusively demonstrated that p53 is a tumor suppressor, as these animals developed malignant tumors within 4-6 months after birth, mainly T-cell lymphomas (about 75%) [26-28], and some soft tissue sarcomas. Surprisingly, considering the key role that p53 plays in multiple cellular processes, $p53^{-/-}$ mice are viable and

developmentally normal (the only exception is a low but increased frequency of exencephaly in female fetuses).

Heterozygous *p53*$^{+/-}$ mice are also highly susceptible to the development of spontaneous tumors, although they display a longer latency to tumor development and develop a different spectrum of tumors compared with *p53*$^{-/-}$ mice [26-29]. Approximately half of the tumors in *p53*$^{+/-}$ mice displayed loss of heterozygosity (LOH) at the *p53* locus, suggesting that complete loss of *p53* is important for tumor development but not strictly required [30]. These results suggest that reduced *p53* gene dosage may be sufficient to promote tumorigenesis, a notion consistent with the finding that p53$^{+/-}$ cells have p53-related phenotypes intermediate between wild-type and *p53*$^{-/-}$ cells [31]. *p53*$^{+/-}$ mice display other special features that make them extremely valuable for cancer research. For example, since *p53*$^{+/-}$ mice are susceptible to carcinogen-induced tumors in a wide array of tissues, they represent an interesting model for carcinogenicity assays. Moreover, *p53*$^{+/-}$ mice provide an excellent model to study the human Li-Fraumeni familial cancer syndrome, since the inheritance of only one mutant p53 allele renders these patients highly susceptible to the development of multiple tumor types early in life [32].

New insight into the mechanisms by which p53 suppresses tumor development has arisen from the crossing of p53 deficient mice with other tumor-susceptible genetically altered murine strains, namely transgenic mice overexpressing oncogenes and knock-out mice defective for other tumor suppressors (Table 2, online Supplement). In short, these studies highlight that both the proapoptotic and antiproliferative activities of p53 can contribute to its tumor suppressor function, but their relative importance seems to be dependent on the specific model system. Thus, available murine models offer valuable tools to further dissect molecular aspects of p53-dependent tumor suppression and to evaluate new therapies.

4. Is accelerated aging a price to be paid for having too much p53?

Several murine models have shown that increasing *p53* copy number or activity can reduce cancer susceptibility. However, in agreement with other evidence suggesting a relationship between the activity of certain oncosuppressors and aging [33], p53 gain-of-function can also provoke accelerated aging in some experimental settings. Tyner et al. obtained mice containing a mutant *p53* allele (*m* allele) generated through an aberrant gene-targeting event [34]. The *m* allele lacks exons 1-6, but contains exons 7-11 under the transcriptional control of the promoter from an upstream gene. At least in vitro, the p53 *m* mutant seems to interact with endogenous wild-type p53 resulting in a moderate increase in its transcriptional activity. Moreover, heterozygous *p53*$^{+/m}$ cells exhibit increased response to ionizing radiation, and *p53*$^{+/m}$ mice (*m* mice) are resistant to spontaneous tumor development. The authors hypothesized that the interaction between *m* mutant and wild-type p53 proteins could convert some of the latent wild-type p53 into a more active conformation. Surprisingly, *m* mice also display accelerated aging and die prematurely. The authors suggested that impaired ability of stem cells to produce progenitors and mature into differentiated cells due to p53-mediated growth inhibition is the underlying cause of premature aging in *m* mice. It is noteworthy however that, in addition to *p53* truncation, the *m* mice have a

deletion of 24 upstream genes, which may contribute to their accelerated aging [35].

Maier et al. generated transgenic mice that ectopically express p44 (Tgp44), a naturally-occurring shorter p53 isoform lacking the main transactivation domain [36] (TDI, Fig. 1A). Strikingly, premature aging is a characteristic of Tgp44 mice, which exhibit an imbalance between p44 and full-length p53 levels that leads to increased (rather than decreased, as might be expected) RNA levels of several p53 transcriptional target genes, such as *p21*, *Mdm2*, and *Ugfbp3*. Therefore, it has been suggested that an overall enhancement of p53 transcriptional activity is the main cause of accelerated aging in Tgp44 mice. However, Gadd45 is not overexpressed in these mice, suggesting that p44 overexpression does not cause a general increase in p53 transcriptional activity. Tgp44 mice also display increased cellular senescence and insulin-like growth factor (IGF) signaling, an observation of special interest since loss-of-function mutations in genes encoding components of the insulin/IGF signaling pathway significantly augment life-span in *Caenorhabditis elegans* and *Drosophila melanogaster* [37]. On the other hand, it has not been ruled out that p44 acts as a dominant negative inhibitor of the p53-related p63 protein. If this were the case, the Tgp44 phenotype might actually be p63 dependent, since $p63^{-/-}$ mice also exhibit premature aging [38].

In contrast to *m* and Tgp44 mice, 'Super-p53' transgenic mice carrying one or two wild-type *p53* transgenes in addition to the two endogenous alleles age normally, presumably because the *p53* transgene is regulated in a physiological manner so that it remains in a latent form in the absence of cellular stress [39]. Remarkably, 'Super-p53' mice exhibit increased p53 activity following carcinogen treatment, have enhanced apoptotic response to DNA damaging irradiation, and are resistant to carcinogen-induced tumors. Recently, it was shown that telomere ablation in 'Super-p53'/telomerase-null mice decreases chromosomal damage compared with 'Wild-type-`p53'/telomerase-null counterparts; however, the presence of extra p53 activity in telomerase-null mice does not affect the rate of age-induced telomere shortening [40].

The $Mdm2^{puro/\Delta 7\text{-}12}$ mouse model also provides evidence that constitutive high p53 activity can lead to tumor suppression without accelerating aging or reducing life span [41, 42]. These mice have one hypomorphic and one null allele of *Mdm2* and express approximately 30% of the wild-type level of Mdm2 protein coincident with constitutively increased p53 function and elevated levels of p53 transcriptional targets in all tissues analyzed. $Mdm2^{puro/\Delta 7\text{-}12}$ mice are small, lymphopenic and radiosensitive, alterations that are completely rescued by deleting p53.

Based on the aforementioned findings, it can be argued that accelerated aging in the Tgp44 [36] and *m* mice [34] might not be due to constitutive hyperactive p53, but to an imbalance in p53 activity derived from the overexpression of truncated forms of p53 lacking more or less of its N-terminus. Indeed, N-terminally truncated forms of p53 can modify the affinity of the full-length protein for specific promoters in a dominant-negative fashion [43]. Given that increased p53 activity can also enhance

tumor resistance without accelerating aging [39, 41], it is of utmost importance to precisely establish under which conditions p53 gain-of-function triggers premature aging (and other potential unwanted effects), and whether this phenotype depends on abnormal p53-dependent transcriptional and/or transcription-independent activation.

5. Pharmacological restoration of p53 transcriptional activity as anticancer therapy

Given that approximately 50% of all human tumors harbor dysfunctional forms of p53, much effort has been devoted to developing small molecules capable of reinstating wild-type p53 transcriptional activity in p53 mutated tumor cells. PRIMA-1 can induce apoptosis in human tumor cells harboring mutant p53 through restitution of p53 transcriptional activity via restoration of sequence-specific DNA binding and the active p53 conformation [44]. Human xenograft studies in nude mice revealed an antitumor effect of PRIMA-1 with no apparent toxicity [44]. Another exciting drug is the styrylquinazoline CP-31398, which causes the accumulation of conformationally active p53 and induces apoptosis and/or growth arrest in cells with mutant p53, and slows down tumor xenograft growth in nude mice [45, 46]. These effects of CP-31398 are likely to result from the restoration of a wild-type-associated epitope on the DNA-binding domain of mutant p53, and from stabilization of p53 via inhibition of its ubiquitination and degradation. Combination of CP-31398 and TRAIL or chemotherapeutic agents enhanced cancer cell death, possibly through upregulation of p53-regulated genes such as KILLER/DR5. However, CP-31398 also has p53-independent effects since it alters the expression of non-p53 target genes in addition to p53-responsive genes [46].

In tumors in which p53 is not mutated, the endogenous wild-type p53 protein can be activated without applying genotoxic stress by freeing it from its inhibitor MDM2 [47]. Chalcones [48] and chlorofusin [49] are unlikely to find clinical application for the treatment of such tumors because of their high IC_{50} for inhibition of the MDM2-p53 interaction, complex chemical structure and potential off-target effects. In contrast, the recently described nutlins and RITA (Reactivation of p53 and Induction of Tumour cell Apoptosis), which can induce p53-dependent growth arrest and apoptosis of tumor cells, appear very attractive [47]. Nutlins are cell-permeable cis-imidazoline derivatives which displace p53 from the hydrophobic pocket of the p53-binding region of MDM2 (but interestingly not of the MDM2 homolog MDMX/4) and thereby disrupt the MDM2-p53 complexes in vitro and in vivo with IC_{50} values in the low nanomolar range [50]. Notably, RITA can induce expression of p53 target genes and massive apoptosis in tumor cell lines expressing wild-type p53 but not in untransformed cells [51]. Nonetheless, controversy exists regarding its mechanism of p53 activation, since Issaeva et al reported that RITA disrupts MDM2-p53 complexes in vitro and in vivo by binding to wild-type p53 [51], but Krajewski et al have reported that this drug does not block the p53-MDM2 interaction [52].

Pharmacological inhibitors of the E3 ubiquitin ligase activity of MDM2 may also find application for treating tumor cells with wild-type p53. Three inhibitors of MDM2-mediated p53 ubiquitination with an IC_{50} in the low

micromolar range and apparently no effect on other ubiquitin ligases or on MDM2-autoubiquitination have been described [53]. HLI98 compounds were selected for their capacity to hinder MDM2 E3 activity via inhibition of autoubiquitination [54]. These drugs lead to the stabilization of both MDM2 and p53, thus inducing p53-dependent transcriptional activity and apoptosis. How HLI98 activates p53 in spite of inducing high levels of MDM2 is not well understood.

Although much work is still needed to develop drugs that efficiently and safely restore wild-type p53 activity, the therapeutic potential of such approach has been highlighted by recent studies demonstrating that p53 restitution can induce regression of different established tumors in mice lacking p53 function [55-57]. These studies also provide proof-of-concept that at least some tumors harbor the signals needed for p53 stabilization and activation, and thus are vulnerable to therapies aimed at restoring or promoting p53 function.

6. Mechanisms of transcription-independent proapoptotic activity of p53 and its therapeutic potential

Besides its classical transcription-dependent activities, evidence is mounting that transcription-independent activities of p53 are also important for its proapoptotic function (Fig. 2). Although the first descriptive reports on this topic date back to 1994 [58, 59], only in the last few years has it become the center of intensive research when a mechanistic basis of action was elucidated. Several related synergistic mechanisms were identified: they link p53 protein to the intrinsic mitochondrial death pathway by direct interaction with anti and proapoptotic members of the BCL family of mitochondrial permeability regulators (Box 1). It was demonstrated that a fraction of induced p53 rapidly translocates to the mitochondrial outer membrane (MOM) early during p53-dependent apoptosis, but not during p53-independent apoptosis [60, 61]. This is a universal p53 response since it occurs in primary, immortal and transformed cultured cells and in normal tissues upon the entire gamut of p53-inducing stresses such as DNA damage, hypoxia and oncogene deregulation [60-62]. Other laboratories confirmed these findings, supporting the existence of a direct p53-mediated mitochondrial death program [63-67]. Very recently, MDM2-dependent monoubiquitylation was shown to promote p53 mitochondrial translocation [68].

A direct apoptogenic role of mitochondrial p53 was first suggested by demonstrating that expression of a mitochondrially-targeted p53 fusion protein - devoid of any residual transciptional activity - efficiently triggered apoptosis in $p53^{-/-}$ cells [60]. Subsequently, mitochondrial p53 was shown to engage in complexes with the antiapoptotic MOM-resident proteins BCL-XL and BCL-2 [69]. This interaction antagonizes the membrane-stabilizing activity of BCL-2 and BCL-XL. The p53-BCL-XL/2 interaction likely releases proapoptotic proteins such as tBID or BAX/BAK from preformed inhibitory complexes with BCL-XL and BCL-2 (Fig. 2A). Purified recombinant p53 added to healthy liver mitochondria causes MOM permeabilization (MOMP) and oligomerization of BAK and BAX, and induces rapid and complete release of potent apoptotic activators like

cytochrome c, SMAC and AIF [65, 69]. Structural analysis confirmed the prediction that the DNA-binding region of p53 and the BH4 domain of BCL-XL are interacting domains in the p53-BCL-XL complex [69, 70]. Conversely, p53's ability to interact with BCL-XL/2 and to induce cytochrome c release is impaired or lost in tumor derived transactivation-deficient missense mutants [69, 71], thus suggesting that at least some tumor-associated mutations, including hotspot mutations, may represent “double hits”, inactivating both nuclear and mitochondrial p53-dependent functions.

A direct interaction between mitochondrial p53 and proapoptotic BAK was also reported [65]. Specifically, p53 competes for BAK interaction with MCL-1, thereby disrupting the antiapoptotic BAK/MCL-1 complex resulting in BAK oligomerization and MOMP (Fig. 2B). Thus, mitochondrial p53 can both neutralize antiapoptotic members as well as activate proapoptotic members of the BCL-2 family. The in vivo functional relevance of mitochondrial p53 might be highlighted by a recent report describing a small molecule named pifithrin-μ (PFTμ), which is reported to selectively inhibit p53 mitochondrial translocation by reducing its affinity to BCL-XL and BCL-2 without interfering with the p53 transcription function. PFTμ strongly reduces γ-radiation-induced thymocyte cell death and rescues irradiated mice from lethal bone marrow failure [72]. If confirmed, PFTμ might represent a promising therapeutic strategy for the treatment of many side effects of radiation and chemotherapy which are mediated by p53-dependent apoptosis in normal sensitive tissues such as bone marrow, lymphoid organs and gut. Moreover, such a strategy might not reduce p53-dependent tumor suppression since the exuberant ‘pathologic’ effect of p53 in normal tissues does not appear to be absolutely essential for the tumor suppressor effect of p53 [73].

Cytosolic p53 can also promote MOMP and apoptosis by directly inducing oligomerization and activation of proapoptotic BAX, which localizes in the cytoplasm of healthy cells [74, 75]. When endogenous p53 immunopurified from DNA-damaged cells was coincubated with recombinant BAX and isolated mitochondria or synthetic liposomes, BAX oligomerization occurred with similar kinetics and concentrations as those produced by the proapoptotic protein tBID, which also induces BAX oligomerization and MOMP. A model was suggested that PUMA, a transcriptional target of p53, couples nuclear and cytosolic p53 functions [76]. In the absence of cellular stress, the low level of nuclear p53 is insufficient to activate PUMA transcription and the small amount of cytosolic p53 is kept inactive via interaction with cytosolic BCL-XL. Death signals, such as UV-induced DNA damage or oncogenic activation, rapidly increase nuclear p53 level, thus triggering transactivation of PUMA, which then binds BCL-XL, hence releasing p53 and activating BAX (Fig. 2C). It was also shown that BAD is transactivated by p53 and forms BAD/p53 complexes at the mitochondria to induce apoptosis [77].

In vivo, mitochondrially-targeted p53 can induce apoptosis and suppress the growth of Burkitt-type primary B-lymphomas that are either p53-null, p53-mutant or ARF-null [78, 79]. Moreover, it was suggested that some of the most promising drugs that target p53, such as Nutlin-3a and PRIMA-1 (see above), exert their proapoptotic effect at least partially in a transcription-

independent manner [75, 80]. Thus, future studies should continue to explore the efficacy and safety of therapeutic strategies based on transcription-independent activities of p53.

7. Future directions

The availability of small compounds that activate p53 function (e.g., Nutlins, RITA, PRIMA-1, and CP-31398) opens new avenues to fight cancer. However, critical issues need to be addressed before these drugs find clinical application (Box 2). First, while p53 reactivation promotes tumor regression in the mouse [55-57], it might favor the growth of p53-resistant tumors carrying inactivating mutations in components of the p53 pathway, as shown in the Eμ-myc mouse lymphoma model [55]. Second, indiscriminate p53 activation in normal cells can lead to fatal pathologies that include ablation of radiosensitive tissues, as was shown in *Mdm2*-null mice [81]. Third, p53 gain-of function in some murine models causes premature aging, possibly due to abnormal p53 activation [34, 36]. Thus, additional studies are needed to optimize p53 activation strategies to achieve tumor suppression while minimizing noxious side effects. Other important issues are to improve selectivity and to establish optimal dose and time of treatment. Restricting delivery of therapeutic agents to solid tumors, as opposed to systemic administration, should be considered. It is also critical to investigate the potential synergistic efficacy of combining p53-activating drugs with standard therapies. Since p53 is ubiquitinated by ubiquitin ligases other than MDM2, effective p53 activation might require the development of additional ubiquitin ligase inhibitors. Finally, additional work is required to unravel the molecular mechanisms underlying transcription-independent functions of mitochondrial/cytosolic p53 and their (patho)physiologic relevance in vivo, since targeting p53 to mitochondria appears effective for treating murine B-lymphomas [78, 79].

8. Concluding remarks

p53 inactivation is a hallmark of most human cancers. Genetic manipulation in the mouse has (1) unequivocally demonstrated the tumor suppressor activity of p53, (2) provided proof-of-principle that p53 gain-of-function can restrain cancer, and (3) demonstrated tumor regression upon p53 reactivation in vivo. In recent years, several promising p53-activating drugs were developed and tested in vitro and in vivo. While it is possible to deter cancer by activating p53, the possibility of noxious side effects has raised some concerns, thus highlighting the need of further developments in this field. In particular, the recent demonstrations that mitochondrial and cytosolic p53 can exert transcription-independent activities and that mitochondrial p53 can suppress murine lymphomas are of great interest. Although further studies are necessary to asses the effectiveness and safety of transcription-independent p53-based strategies in different cancer models, this approach might avoid some of the unwanted side effects associated with 'conventional' p53 gain-of-function methods. Moreover, mitochondrial p53 exploits the shortest possible circuit to cell death and thus might be unaffected by abnormal genomic silencing. This frequently occurs in tumors and may minimize the therapeutic efficacy of strategies aimed at increasing p53

transcriptional activity. As our knowledge on classical and novel functions of p53 grows, the challenge will be to translate all this information into safe anticancer therapies for humans.

9. Acknowledgements

We thank María J. Andrés-Manzano for the preparation of figures. Work in the laboratory of V.A. is supported in part by grants from Ministerio de Sanidad y Consumo/Instituto de Salud Carlos III (Red Temática de Investigación Cooperativa en Enfermedades Cardiovasculares RECAVA), from Sociedad Española de Cardiología, and from Ministerio de Educación y Ciencia and the European Regional Development Fund (SAF2004-03057). J.J.F. is supported by a CSIC-I3P program predoctoral fellowship cosponsored by the European Social Fund.

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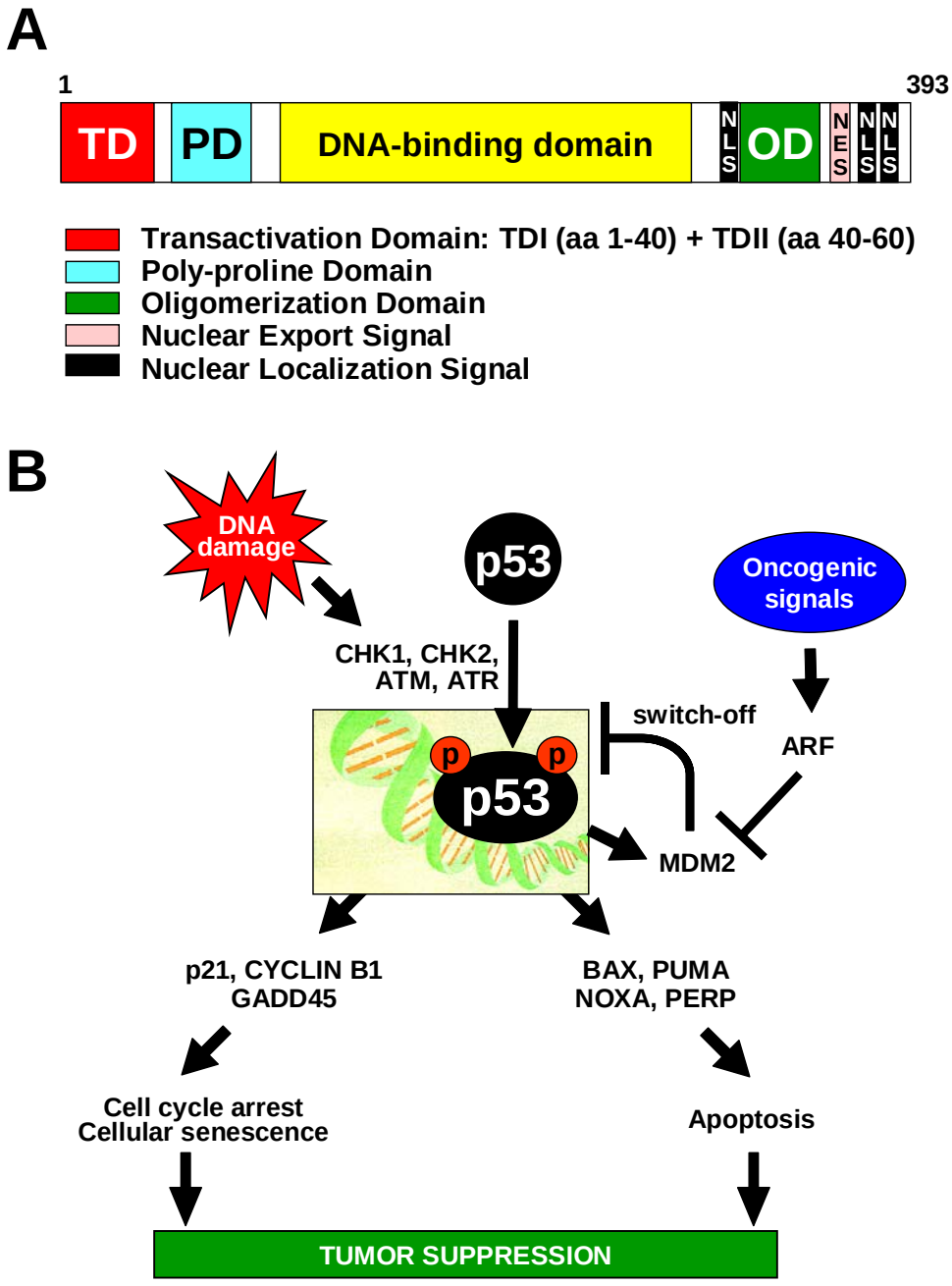

Fig. 1: Transcription-dependent pathways of p53-mediated growth arrest and apoptosis. (A) Structural and functional domains of p53. The transactivation domain includes a main TDI and a secondary TDII. **(B)** p53 transcriptional activity can be induced by different forms of cellular stress. For example, DNA damage stabilizes and activates p53 mainly through covalent modifications of p53 itself or of its main inhibitor MDM2. On the other hand, p53 activation by oncogenic signalling is executed mainly via $p19^{Arf}$-dependent inhibition of MDM2. Notably, MDM2 is a transcriptional target of p53, thus establishing an autoregulatory loop. Once activated, p53 can modulate the transcription of genes involved in the control of cell cycle progression and apoptosis, thus playing a key role in tumor suppression.

Figure 2

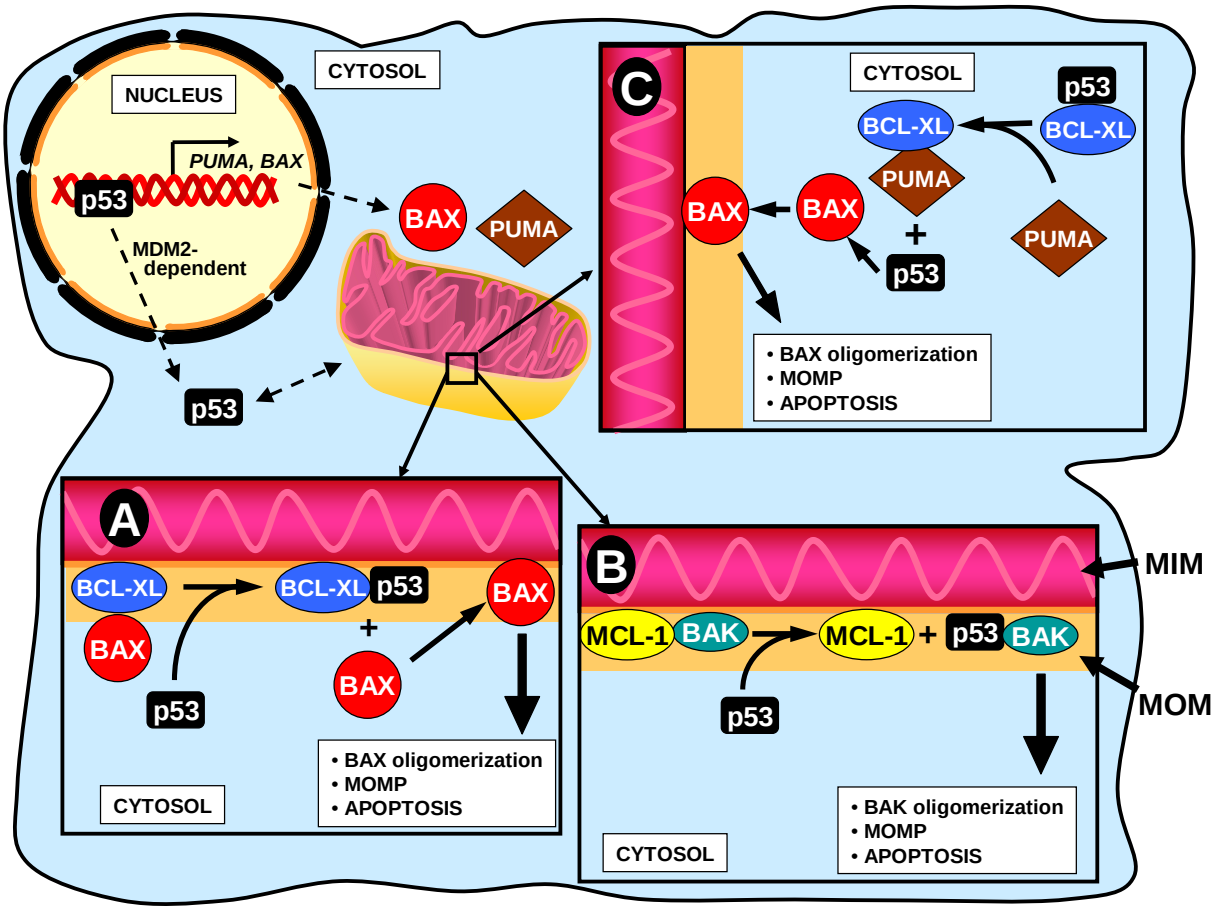

Fig. 2: Mitochondrial and cytosolic transcription-independent proapoptotic activities of p53. (A) Activated p53 can translocate to the mitochondrial outer membrane and promote apoptosis by interacting with BCL-XL, hence releasing proapoptotic factors, such as tBID and BAX [69]. **(B)** Mitochondrial p53 can also release BAK from inhibitory BAK-MCL-1 complexes, thus allowing BAK-dependent apoptotic cell death [65]. **(C)** The p53 target PUMA can release p53 from inhibitory p53-BCL-XL complexes. Cytosolic p53 can then promote mitochondrial translocation of BAX and apoptosis [74-76]. The cytosol is shown in blue, the mitochondrial inner membrane (MIM) in pink and the mitochondrial outer membrane (MOM) in orange. MOMP: MOM permeabilization.

Box 1. The BCL family of proteins

The BCL family of apoptosis regulators comprises three main subfamilies:

1) Proteins that sequester proapoptotic family members thus inhibiting the mitochondrial apoptotic pathway (e. g., BCL-2, BCL-XL, and MCL1).

2) Proteins that directly induce mitochondrial outer membrane permeabilization (MOMP) and apoptosis (e. g., BAX and BAK).

3) BH3-only proteins, which includes the so-called 'activators' (e.g., BIM and BID) that directly bind and activate BAX and BAK, and 'enablers' (e.g., BAD and BIK), which bind the antiapoptotic family members to release the 'activators'.

This classification is also closely related to the structure and the sequence similarity among these proteins. BCL proteins possess up to four conserved BCL-2 homology (BH) domains dubbed BH1, BH2, BH3, and BH4. Most of the anti-apoptotic members of the family, such as BCL-2 and BCL-XL, display sequence conservation in all four domains. In contrast, the pro-apoptotic molecules which directly induce MOMP (e.g., BAX and BAK) frequently only exhibit high conservation of BH1, BH2 and BH3. Finally, BH3-only proteins display sequence similarity only in the BH3 domain, which seems to be a critical cell death domain of the proapoptotic BCL family members.

Box 2. Outstanding questions

Several unresolved issues need to be addressed before p53-targeted therapies find clinical application.

- **Premature aging**. It is necessary to precisely establish under which conditions p53 gain-of-function triggers premature aging, as has been shown in some but not all murine models, and whether this phenotype depends upon abnormal p53-dependent activation. Moreover, animal studies should address whether accelerated aging is a consequence of prolonged treatment with p53 activating drugs (e.g., Nutlins, RITA, PRIMA-1, and CP-31398).
- **Unwanted side effects in normal tissues**. Mouse studies have shown that p53 activation in normal tissues can lead to fatal pathologies, thus narrowing the therapeutic window of p53 activators. Future studies should improve selectivity and establish optimal dose and time of treatment to achieve tumor suppression while minimizing noxious side effects.
- **Combination therapies**. It is important to address the potential synergistic efficacy of novel p53-activating strategies in combination with standard therapies.
- **Appearance of p53-resistant tumors**. Prolonged treatment with p53-activating drugs might favor the development of tumors carrying inactivating mutations in components of the p53 pathway. In the Eμ-myc mouse lymphoma model, reactivation of p53 function potently selects for emergence of p53-resistant tumors through inactivation of $p19^{Arf}$ or p53 itself.
- **Therapeutic potential of transcription-independent p53 activities**. Targeting p53 to the mitochondria has already shown therapeutic efficacy for the treatment of murine B-lymphomas in vivo. Whether this approach is effective and safe in other cancer settings remains to be investigated.

Supplementary data

Classic and novel roles of p53: prospects for anticancer therapy

José J. Fuster[1], Silvia M. Sanz-González[1], Ute M. Moll[2] and Vicente Andrés[1]

[1]Vascular Biology Unit, Instituto de Biomedicina de Valencia (IBV-CSIC), Spanish Council for Scientific Research, 46010 Valencia, Spain
[2]Department of Pathology, State University of New York at Stony Brook, Stony Brook, NY 11794-8691, USA
Corresponding author: Andrés, V. (vandres@ibv.csic.es).

Table S1. Examples of human genes exhibiting p53-dependent transcriptional regulation

Cellular process	Gene	GenBank ID	Refs
Apoptosis, autophagy and survival	*APAF1*	317	[1–3]
	BAX	581	[4]
	BID	637	[5]
	FAS	355	[6]
	DRAM	55332	[7]
	FDXR (Ferredoxin reductase)	2232	[8]
	IGFBP3	3486	[9]
	KILLER/DR5	8795	[10]
	NOXA	5366	[11]
	p53AIP1	63970	[12]
	p53DINP1	94241	[13]
	WIG1/PAG608	64393	[14]
	PERP	64065	[15]
	PIDD	55367	[16]
	PIG3	9540	[17–19]
	PIG8/EI24	9538	[17,20,21]
	PTEN	5728	[22]
	PUMA	27113	[23,24]
	TIGAR	57103	[25]
	WIP1	8493	[26]
Cell-cycle regulation and DNA repair	*BTG2*	7832	[27]
	CDKN1A ($p21^{Cip1}$)	1026	[28]
	14-3-3-σ	7532	[29]
	GADD45	1647	[30]
	p53R2	50484	[31,32]
Angiogenesis and metastasis	*KAI1*	3732	[33]
	MMP2	4313	[34]
	MASPIN	5268	[35]
	P4HA2	8974	[36]
	TSP1	7057	[37]
Energy metabolism	*TIGAR*	57103	[25]
	SCO2	9997	[38]
Autoregulation of p53	*MDM2*	4193	[39,40]
	COP1	64326	[41]
	PIRH2	25898	[42]
	TP73	7161	[43,44]
	CCNG1 (Cyclin G1)	900	[45–47]

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TABLE S2. Murine models to study the interaction between p53 and additional oncoregulatory genes

A. p53 deficiency imposed in oncogene-overexpressing mice.

B. p53 deficiency imposed in mice lacking additional tumour suppressor genes.

A. p53 DEFICIENCY IN ONCOGENE-OVEREXPRESSING MICE		
Compound mutant	**Effects on tumor development[a]**	
MMTV–*Wnt1p53*$^{-/-}$ Overexpression of Wnt1 restricted to mammary glands and salivary glands in a p53-nullizygous background	Cooperativity: reduced latency of mammary tumors. Increased genomic instability and enhanced proliferation. Low levels of apoptosis unaffected by p53 status	[1,2]
MMTV–*Ha-rasp53*$^{-/-}$ Overexpression of Ha-Ras restricted to mammary glands and salivary glands in a p53-nullizygous background	Tumor-type specific cooperativity: higher frequency of salivary tumors, but other tumors unaffected. Defective cell-cycle arrest. Low levels of apoptosis unaffected by p53 status	[3]
Cd2–*c-myc p53*$^{-/-}$ Overexpression of c-Myc restricted to T cells in a p53-nullizygous background	Cooperativity: increased frequency and reduced latency of T-cell lymphomas	[4]
Eμ–*c-myc p53*$^{+/-}$ Overexpression of c-Myc restricted to T cells in a p53-deficient heterozygous background	Cooperativity: accelerated development of B-cell lymphomas. Enhanced proliferation. Apoptosis unaffected by p53 status	[5]
Eμ–*c-myc p53*$^{+/-}$ Overexpression of c-Myc restricted to T cells in a p53-deficient heterozygous background	Cooperativity: accelerated development of B-cell lymphomas. Decreased apoptosis and increased genomic instability upon LOH[b]. Proliferation unaffected by p53 status	[6]
MMTV–*c-myc p53*$^{+/-}$ Overexpression of c-Myc restricted to mammary glands and salivary glands in a p53-deficient heterozygous background	Tumor-type specific cooperativity: shorter latency of T-cell lymphomas, but unaltered latency of mammary carcinomas.	[7]
Cd2–*Scl*/*Tal1p53*$^{+/-}$ Overexpressión of Scl restricted to T cells in a p53-deficient heterozygous background	Cooperativity: accelerated development of T-cell lymphomas	[8]
CD2–*Scl*/*Tal1p53*$^{-/-}$ Overexpressión of Scl restricted to T cells in a p53-nullizygous background	Absence of cooperativity. Pattern of organ involvement shifted from central to peripheral lymphoid organs	[8]
Lck–*Scl*/*Tal1 p53*$^{+/-}$ Overexpression of Scl restricted to lymphoid tissues in a p53-deficient heterozygous background	Cooperativity: accelerated development of T-cell lymphomas	[9]
***Bcl-2*–Ig *p53*$^{-/-}$** Overexpression of Bcl-2 restricted to T-cells in a p53-nullizygous background	5 Absence of cooperativity	[10]

B. p53 DEFICIENCY IN MICE LACKING ADDITIONAL TUMOR SUPPRESSORS		
Compound mutant	**Effects on tumor development[a]**	
***Rb*$^{+/-}$*p53*$^{+/-}$** Rb heterozygosity in a p53-deficient heterozygous background	Cooperativity: development of pancreatic tumors, thyroid carcinomas and pinealoblastomas (not observed in single mutants)	[11]
***Ap*$^{+/-}$*p53*$^{-/-}$** APC heterozygosity in a p53-deficient heterozygous background	Tumor-type specific cooperativity: development of pancreatic tumors (not observed in single mutants), but unaffected intestinal tumor development (characteristic of APC deficiency)	[12]
Nf1*$^{+/-}$ *p53*$^{+/-}$ *cis Nf1 heterozygosity in a p53-deficient heterozygous background (wild-type alleles of Nf1 and p53 on the same chromosome 11)	Cooperativity: increased incidence of soft-tissues sarcomas; development of malignant peripheral nerve sheath tumors (not detected in single mutants). Reduced survival related to single mutants or *trans* double mutants. LOH[b] for both genes in tumors	[13–15]
Nf1*$^{+/-}$ *p53*$^{+/-}$ *trans Nf1 heterozygosity in a p53-deficient heterozygous background (wild-type alleles of Nf1 and p53 on opposite chromosomes 11)	Absence of cooperativity, possibly related to LOH[b] for either Nf1 or p53 in tumors, but not simultaneously for both	[13–15]
Nf2*$^{+/-}$ *p53*$^{+/-}$ *cis Nf2 heterozygosity in a p53-deficient heterozygous background (wild-type alleles of Nf1 and p53 on the same chromosome 11)	Cooperativity: higher incidence of osteosarcomas, predominantly nasal osteosarcomas (rarely seen in Nf2$^{+/-}$ mice). Reduced survival related to single mutants or *trans* double mutants. LOH[b] for both loci in tumors	[16]
Nf2*$^{+/-}$ *p53*$^{+/-}$ *trans Nf2 heterozygosity in a p53-deficient heterozygous background (wild-type alleles of Nf1 and p53 on opposite chromosomes 11)	Cooperativity: higher incidence of osteosarcomas. LOHb for both loci in tumors	[16]
***Brca1*$^{+/-}$ *p53*$^{-/-}$** BRCA1 heterozygosity in a p53-nullizygous background	Cooperativity: increased incidence of mammary tumors	[17]
***Brca1* Ko/Co MMTV–Cre *p53*$^{+/-}$** Brca1 nullizygosity restricted to mammary glands in a p53-deficient heterozygous background	Cooperativity: accelerated formation of mammary tumors	[18]
***Brca1*$^{\Delta 11/\Delta 11}$ *p53* $^{+/-}$** Brca1 nullizygosity in a p53-deficient heterozygous background	Cooperativity: accelerated formation of mammary tumors with p53 LOH[b]. Rescue of the embryonic lethality of BRCA1$^{\Delta 11/\Delta 11}$ mice. Premature cell senescence and accelerated aging	[19,20]
***Brca2* F11/11 K14-Cre/Tr*p53* F2-10/F2-10** Brca2 and p53 inactivation restricted to skin and mammary glands	Cooperativity: higher incidence of mammary and skin tumors	[21]

[a] Cooperativity refers to increased incidence or accelerated development of tumors in compound mutant compared to single mutant mice.

[b] Loss of heterozygosity

References Table S2

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