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Cytoplasmic functions of the tumour suppressor p53

Douglas R. Green[1,2] & Guido Kroemer[3,4]

The principal tumour-suppressor protein, p53, accumulates in cells in response to DNA damage, oncogene activation and other stresses. It acts as a nuclear transcription factor that transactivates genes involved in apoptosis, cell cycle regulation and numerous other processes. An emerging area of research unravels additional activities of p53 in the cytoplasm, where it triggers apoptosis and inhibits autophagy. These previously unknown functions contribute to the mission of p53 as a tumour suppressor.

Transcriptional and non-transcriptional effects of p53

Approximately half of human cancers have inactivating mutations of p53 (known as TP53 in human), and most of the remaining malignancies deactivate the p53 pathway by increasing its inhibitors, reducing its activators or inactivating its downstream targets. p53 is best characterized as a transcription factor that binds to specific DNA sequences and transactivates a number of genes with a variety of functions including cell cycle arrest, apoptosis, causing changes in metabolism and others[1] (Fig. 1). In addition to this nuclear activity, p53 also possesses biological activities that are cytosolic and transcription-independent. Several years ago it was noted[2] that overexpression of a mutant p53, lacking most of the DNA-binding domain (DBD) and completely deficient in transactivation function, could nonetheless trigger apoptosis. Indeed, overexpression of a variety of transactivation-incompetent p53 mutants can efficiently induce apoptosis in human cells[3]. Consistent with this, it was found[4] that apoptosis induced by stabilization of an ectopically expressed temperature-sensitive mutant of p53 could proceed in the absence of RNA and protein synthesis. Similarly, activation of p53 was found to trigger apoptosis even in the absence of a nucleus[5]. p53-reactivating drugs that interact with oncogenic, mutant p53 protein causing it to adopt a wild-type conformation can induce apoptosis under conditions of complete transcriptional or translational blockade[5,6]. Most recently, mice were generated in which the endogenous *p53* (also known as *Trp53* in mouse) gene was replaced by a chimaeric p53 protein that is capable of transactivation yet lacks several domains that are required for other p53 functions[7]. In fibroblasts from such mice, this chimaeric p53 protein was transcriptionally active and able to induce cellular senescence, but was unable to trigger apoptosis[7]. Together, these observations support the idea that a cytoplasmic pool of p53 can induce apoptosis through a transactivation-independent mechanism.

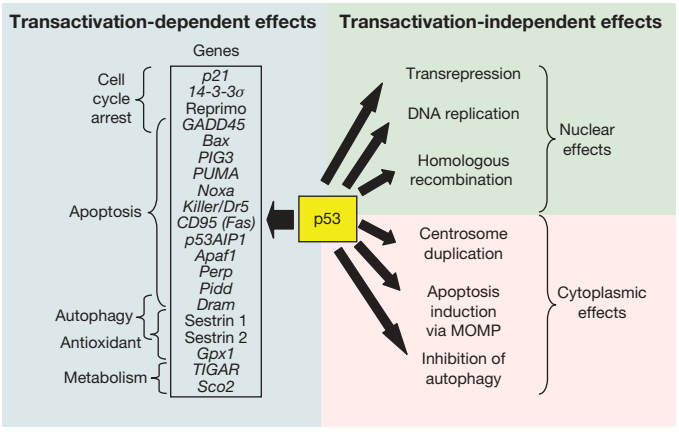

Figure 1 | Classification of p53 activities. On the left side, some genes that are transactivated by p53 are exemplified, together with a few of the functional consequences of p53 activation. On the right side, transactivation-independent effects of p53 are listed. These can be divided into nuclear and extra-nuclear (cytoplasmic) p53 activities.

p53 is at the hub of numerous signalling pathways triggered by a range of cellular stresses including DNA damage by exogenous mutagens, oncogene activation, telomere erosion and hypoxia, all of which influence the abundance, subcellular localization, post-translational modification and/or interaction of p53 with cofactors. As a result of these context-dependent, damage-elicited alterations, p53 can facilitate the transient adaptation of cells to stressful conditions, for example, by increasing DNA repair upon a transient cell cycle arrest or by enhancing the expression of enzymes that detoxify reactive oxygen species. Alternatively, p53 suppresses oncogenic potential by mediating an irreversible arrest of the cell cycle or by triggering apoptotic cell death[8]. In addition to its role as a tumour suppressor, p53 has a major role in ageing[9], as well as in the unwarranted loss of post-mitotic cells such as heart muscle cells in infarction and neurons in stroke[10]. It is likely that the biological effects of p53 represent the combined activities of the nuclear and cytoplasmic protein.

Effects of cytoplasmic p53 on mitochondria

The mitochondrial membrane constitutes the battleground on which pro- and anti-apoptotic factors induce or prevent a potentially lethal permeabilization step[11]. Under a variety of cell-death-inducing conditions, p53 rapidly moves to the mitochondria. For instance, whole-body irradiation of mice causes a fraction of cellular p53 to associate with the outer mitochondrial membrane[12]. Similarly, ischaemic damage of the rat brain triggers the translocation of p53 (also known as Tp53 in rat) to the mitochondria of neurons that are particularly vulnerable to hypoxia within the CA1 area of the hippocampus[10]. Once at the mitochondrion, p53 induces mitochondrial outer membrane permeabilization (MOMP), thereby triggering the release of pro-apoptotic factors from the mitochondrial intermembrane space.

[1]Department of Immunology, St Jude Children's Research Hospital, Memphis, Tennessee 38105, USA. [2]Institut Gustave Roussy, [3]Université Paris Sud, Paris 11, [4]INSERM, U848, F-94805 Villejuif, France.

Indeed, mitochondrion-targeted p53 can be as efficient as wild-type p53 in inhibiting tumour growth[12].

Multiple mechanisms have been invoked to explain how p53 triggers MOMP[12]. MOMP is usually inhibited by anti-apoptotic multidomain proteins of the Bcl2 family (such as Bcl2, Bcl-X_L (also known as Bcl2l1) and Mcl1), and is conditional on pro-apoptotic multidomain proteins from the same family (in particular Bax and Bak (Bak1)) that can homo-oligomerize within the outer mitochondrial membrane to form MOMP-mediating supramolecular structures. Depending on their particular affinities for multidomain Bcl2 family proteins, a set of distinct pro-apoptotic 'BH3-only' proteins can directly interact with Bax or Bak to trigger their homo-oligomerization and hence MOMP (these BH3-only proteins are referred to as 'direct activators') and/or neutralize one or more anti-apoptotic multidomain proteins (referred to as 'sensitizers' or 'de-repressors'). In contrast to direct activators (in particularly the proteins Bim (Bcl2l11) and Bid), sensitizers cannot trigger MOMP on their own (because they simply antagonize its inhibition) and require the input of additional stimuli for apoptosis induction[13].

p53 has been suggested to act like a BH3-only protein, either as a direct activator of Bax and/or Bak or as a de-repressor. Under pro-apoptotic conditions, p53 can be co-immunoprecipitated with Bcl2, Bcl-X_L and Bak[12,14,15]. In a defined system involving only recombinant proteins and synthetic membranes, p53 can trigger Bax to permeabilize liposomes through a 'hit and run' mechanism—that is, through transient molecular associations[16]. NMR structures of p53 in a complex with Bcl2 or Bcl-X_L indicate that the DBD of p53 is involved in docking with Bcl2 family proteins[12,17], further supported by biochemical studies on p53 and Bak[18]. Hence, oncogenic p53 mutations affecting the DBD can operate as 'dual hits' and simultaneously abrogate transactivation and direct MOMP induction by p53. In addition, other studies show that the proline-rich region neighbouring the DBD is important for Bax activation[16] and can also associate with anti-apoptotic Bcl2 proteins[18] Further, the p53-binding interfaces within Bcl-X_L and Bak may be distinct, as determined by biochemical approaches[17]. Because p53 has a higher affinity for Bcl-X_L than for Bak[19], it may first engage in molecular interactions with anti-apoptotic Bcl2 proteins (as a sensitizer) and then with Bak and Bax (as a direct activator). Another nuclear transcription factor, Nur77, has previously been shown to bind to Bcl2 in a way that radically changes its conformation, apparently converting Bcl2 into a pro-apoptotic protein[20]. However, the structural changes that p53 imposes on Bcl2 or Bcl-X_L are minor[12,19], indicating that p53 does not function in this manner.

The pro-apoptotic effects of cytoplasmic p53 are not dependent on transcription, in principle. However, the control of transcription by nuclear p53 decisively contributes to the function of cytoplasmic p53. As discussed in more detail below, the p53 target Mdm2 is essential for post-translational regulation of p53, without which the system would not be responsive to cellular stress. Another p53 target, PUMA (Bbc3), controls the sequestration of cytoplasmic p53 by the anti-apoptotic Bcl-X_L protein, releasing p53 to activate Bax[21]. Therefore, without transcription, regulated by nuclear p53, endogenous cytoplasmic p53 may not function (Fig. 2).

One p53-targeted drug, pifithrin-μ, inhibits the pro-apoptotic effects of cytosolic p53, but has no apparent effect on p53-dependent transactivation[22]. This drug blocks the interaction of p53 with Bcl-X_L[22], and probably also the interaction of p53 with pro-apoptotic Bcl2 family members (Bax and Bak), thereby accounting for its anti-apoptotic effects. Pifithrin-μ can rescue mice from otherwise lethal irradiation[22], indicating that selective inhibition of the cytoplasmic p53 pathway is sufficient for radioprotection *in vivo*. Conversely, p53-activating drugs such as CP-31398 induce p53 translocation to mitochondria, as well as p53-dependent MOMP. This p53 translocation can be inhibited by cyclosporin A (CsA)[6], pointing to a possible molecular crosstalk between the CsA inhibitable mitochondrial permeability transition pore and the p53 system. Reportedly,

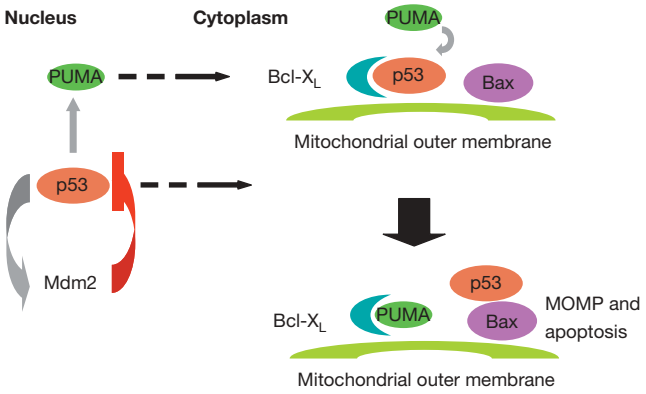

Figure 2 | Interplay of the nuclear and cytoplasmic functions of p53 in apoptosis. Nuclear p53 induces the expression of Mdm2, which acts to inhibit the protein through binding and ubiquitinylation. Cellular stress signals interrupt this inhibition, allowing p53 to accumulate both in the nucleus and in the cytoplasm. In the latter, p53 is sequestered by anti-apoptotic Bcl2 proteins such as Bcl-X_L. Another target of nuclear p53, PUMA, functions to disrupt the Bcl-X_L–p53 interaction. The released p53 can now trigger MOMP and apoptosis through interaction with, for example, Bax.

recombinant p53 may induce a more complete MOMP (with release of the pro-apoptotic factor AIF (Aifm1)) than recombinant Bid added to purified mitochondria *in vitro*[23], pointing to possible differences in mitochondrial permeabilization by p53 and BH3-only proteins.

Inhibition of autophagy by cytoplasmic p53

Macroautophagy (referred to as autophagy) is the sequestration and subsequent digestion of parts of the cytoplasm, allowing for the adaptation of cells to stressful conditions, as well as the removal of damaged, potentially harmful cytoplasmic organelles. Enhanced autophagy, which frequently accompanies cell death, likewise constitutes a failed attempt to adapt to stress and to survive, rather than a lethal catabolic process[24]. Because autophagy has an essential role in the maintenance of genomic stability[25], inhibition of autophagy is oncogenic. Accordingly, loss of only one allele of either of the two haploinsufficient autophagy genes beclin 1 (*Becn1*) or *Uvrag* is sufficient to promote carcinogenesis, and multiple oncogenes including Bcl2, Akt (Akt1) and PI(3)K inhibit autophagy. Similarly, the inactivation of tumour-suppressor proteins such as Pten, Tsc1, Tsc2 and Lkb1 (also known as Stk11) results in autophagy inhibition[24].

Although p53 can transactivate genes that induce autophagy (such as DRAM and sestrins 1 and 2)[26,27], normal levels of p53 mediate a tonic inhibition of autophagy (Fig. 1). In fact, the deletion, depletion or pharmacological inhibition of p53 with pifithrin-α induces autophagy in mouse, human and nematode cells[28]. Suppression of autophagy is mediated by cytoplasmic, not nuclear, p53, and physiological inducers of autophagy (such as nutrient depletion) must destroy the pool of cytoplasmic p53 to induce autophagy[28]. Thus, inhibition of the ubiquitin E3 ligase Mdm2, which targets p53 for destruction, can suppress the induction of autophagy by starvation, rapamycin, lithium or damage of the endoplasmic reticulum[28]. Cytoplasmic (but not nuclear) p53 inhibits the AMP-dependent kinase, a positive regulator of autophagy, and activates mammalian target of rapamycin (mTOR, also known as Frap1), a negative regulator of autophagy[28]. How these effects are achieved, however, remains an open conundrum. Nevertheless, it is not unlikely that the transactivation-dependent metabolic effects of p53 and its cytosolic, transcription-independent inhibition of autophagy cooperate to ensure a coordinated action of p53 in cellular adaptation, such as in reprogramming metabolism towards oxidative phosphorylation.

It is tempting to speculate that the dual action of cytoplasmic p53—inhibition of autophagy and induction of MOMP—may constitute a coordinated response for cell death induction (Fig. 3). Autophagy

accounts for the removal of damaged and permeabilized mitochondria and counteracts the lethal effect of MOMP[29]. Therefore, autophagy inhibition by p53 may further facilitate cell death execution by MOMP. Nonetheless, it is not clear through which mechanisms cytoplasmic p53 switches from its baseline function (autophagy inhibition) to its killer activity (translocation to mitochondria and MOMP induction).

At first glance it appears paradoxical that normal levels of cytoplasmic p53 inhibit autophagy, although inhibition of autophagy is often associated with oncogenesis. This paradox is resolved by the observation that mutant p53 protein, which has lost its transactivation function and which accumulates in the cytoplasm of tumour cells, efficiently inhibits autophagy[30]. The structural features of p53 required for its cytoplasmic pro-apoptotic and anti-autophagic functions are clearly distinct. Indeed, deletion of the DBD does not affect autophagy inhibition by p53. Moreover, point mutations that affect the nuclear functions of p53, as well as its interaction with Bcl2 family proteins, do not abolish its capacity to inhibit autophagy[30]. This may contribute to the strong oncogenic action of certain p53 mutants that is difficult to explain by the mere abolition of their tumour-suppressive capabilities.

Regulating the regulator

The nuclear versus cytoplasmic effects of p53 are determined by multiple post-translational modifications that affect its interaction with other proteins, its shuttling between the cytoplasm and the nucleus and its biological activities. Poly(ADP)ribosylation of p53 leads to its nuclear accumulation[8]. In contrast, monoubiquitylation by Mdm2 stimulates the nuclear export of p53, which on arrival at mitochondria is deubiquitylated by mitochondrial HAUSP, thus generating the apoptotically active non-ubiquitylated p53 (ref. 31). Other post-translational modifications of p53 (such as phosphorylation of carboxy-terminal serines) can stimulate nuclear export and/or mitochondrial association. Moreover, the transcription factor Foxo3a (Foxo3) promotes p53 cytoplasmic accumulation by increasing its nuclear export, hence stimulating direct, p53-mediated MOMP induction[32]. This indicates that the entire context of post-transcriptional p53 modifications and protein interactions can affect the precise subcellular localization and function of p53.

A previously unknown class of p53 activators, the tenovins, activate the tumour-suppressive function of p53 by inhibiting the sirtuins Sirt1 and Sirt2 (ref. 33)—p53 deacetylases—in tumour cells. Similarly, a tumour suppressor, Dbc1 (deleted in breast cancer 1), acts as an endogenous inhibitor of Sirt1 and as a positive regulator of p53 (ref. 34). However, according to one report[35], nuclear p53 is acetylated whereas cytoplasmic p53 is deacetylated. Knockout of Sirt1 facilitates the reactive-oxygen-species-induced nuclear translocation of p53 and simultaneously inhibits direct MOMP induction by cytoplasmic p53, at least in mouse embryonic stem cells[35]. Future investigations must resolve this apparent contradiction to understand which post-transcriptional modifications of p53 determine its pro-MOMP and anti-autophagy activities.

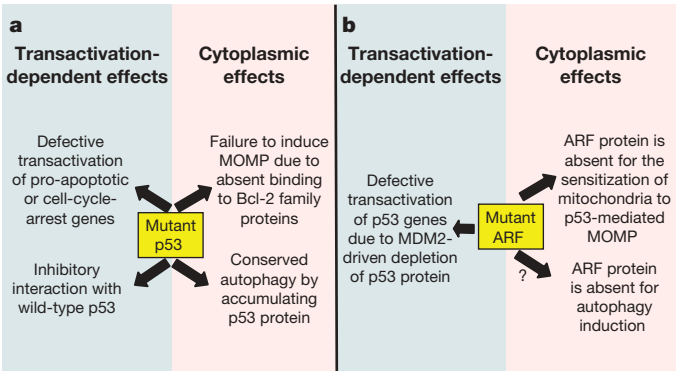

Figure 3 | Concerted oncogenic actions of mutant p53 or inactive ARF in the nucleus and cytoplasm of cancer cells. Hotspot mutations of p53 affecting the DBD can abolish the transactivation of p53 genes as well as the mitochondrion-permeabilizing action of p53, yet leave intact autophagy inhibition by p53 (**a**). Similarly, oncogenic mutations that affect the C terminus of ARF can lead to the depletion of p53 protein, as well as to the abolition of mitochondrion-permeabilizing and autophagy-inducing activities mediated by the ARF protein (**b**).

Future directions and perspectives

Cancer cells are characterized by failing cell cycle checkpoints, reduced propensity to apoptosis and suppressed autophagy. 'Hotspot' mutations of p53 within the DBD usually abolish the transactivation function of p53 (and often create dominant-negative inhibitors of wild-type p53, with which they form heterotetramers), thereby preventing the expression of cell-cycle-arresting, pro-apoptotic and autophagy-inducing genes. Such p53 mutations within the DBD also affect the cytoplasmic functions of p53, reducing its capacity to induce MOMP (through failure to interact with Bcl2 family proteins)[12,15,17], yet leaving intact its inhibitory effect on autophagy[30]. Whether and how such mutations perturb the cytoplasmic regulation of cell cycle checkpoints remains elusive. However, it appears that frequent p53 mutations can contribute to oncogenesis through the concerted subversion of both the nuclear and cytoplasmic programs of tumour suppression (Fig. 3). Similarly, the inactivation of the oncosuppressor Arf (Cdkn2a; which, through the activation of Mdm2, leads to p53 depletion) combines transcriptional effects (due to the absence of p53) with cytoplasmic ones. Indeed, distinct splice variants of Arf can induce MOMP (though through a mechanism distinct from that of p53, involving an interaction with a specific mitochondrial receptor, p32 (C1qbp), or an interaction with Bcl-X_L) and stimulate autophagy[36–38]. Therefore, the net result of Arf inactivation may also be a combined subversion of apoptosis and autophagy, both at the nuclear and at the extra-nuclear levels. ARC (Nol3), an apoptosis-inhibitory protein that is overexpressed in numerous cancers, is present in the nucleus, in which it inhibits p53 tetramerization and stimulates its export, as well as in the cytoplasm, where it neutralizes Bax to inhibit cell death[39]. It remains an open question through which mechanisms many other oncogenic perturbations, such as constitutive activation of the insulin receptor pathway, may affect (p53-dependent?) tumour suppression in the cytoplasm. Understanding the extra-nuclear activities of p53 will likewise furnish new opportunities to pharmacologically modulate the p53 system.

p53 has a prominent—and controversial—role in the regulation of ageing and longevity[9]. In the nematode *Caenorhabditis elegans*, knockout of the p53 orthologue *cep-1* fails to cause oncogenesis, yet significantly increases both median and maximum lifespan. This gain of longevity is lost when autophagy is inhibited[40]. We anticipate that the investigation of whether and how p53 can participate in a longevity-increasing pathway that links apoptosis, caloric restriction, activation of sirtuins and regulation of autophagy will yield crucial insights into the intricate relationship between tumour suppression and ageing that dictates our inexorable, yet variable, fate.

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