



# Switching on p53: an essential role for protein phosphorylation?

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## Abstract

The p53 tumour suppressor protein coordinates widespread changes in gene expression in response to a range of stress stimuli. p53 is regulated primarily through ubiquitylation and protein turnover mediated by its transcriptional target, MDM2. Induction and activation of p53 is achieved largely through uncoupling the p53/MDM2 interaction, with various stress stimuli employing different but overlapping mechanisms to achieve this. p53 undergoes a range of post-translational modifications including multi-site phosphorylation, acetylation, methylation and ubiquitylation. DNA damage pathways in particular engender a large number of phosphorylation events, both in p53 itself and in regulatory partners including MDM2 and MDM4; these modifications mediate both the induction of p53 and stimulation of its activity. Surprisingly, other p53-activating stimuli do not promote multi-site phosphorylation. Moreover, simply uncoupling p53 and MDM2 pharmacologically can induce a robust p53 response. Various lines of evidence propose that activation of p53 via the DNA damage pathways is dispensable for p53-mediated tumour suppression and, by implication, that phosphorylation is not required. In contrast to this view, however, emerging evidence from animal models indicates that phosphorylation may indeed impact on tumour suppression, albeit in a possibly selective manner. Here we review the role of phosphorylation in regulating the p53 response in comparison to mechanisms employed by other stress signalling pathways. We consider its effects on biological outcome and reflect on issues that have yet to be addressed.

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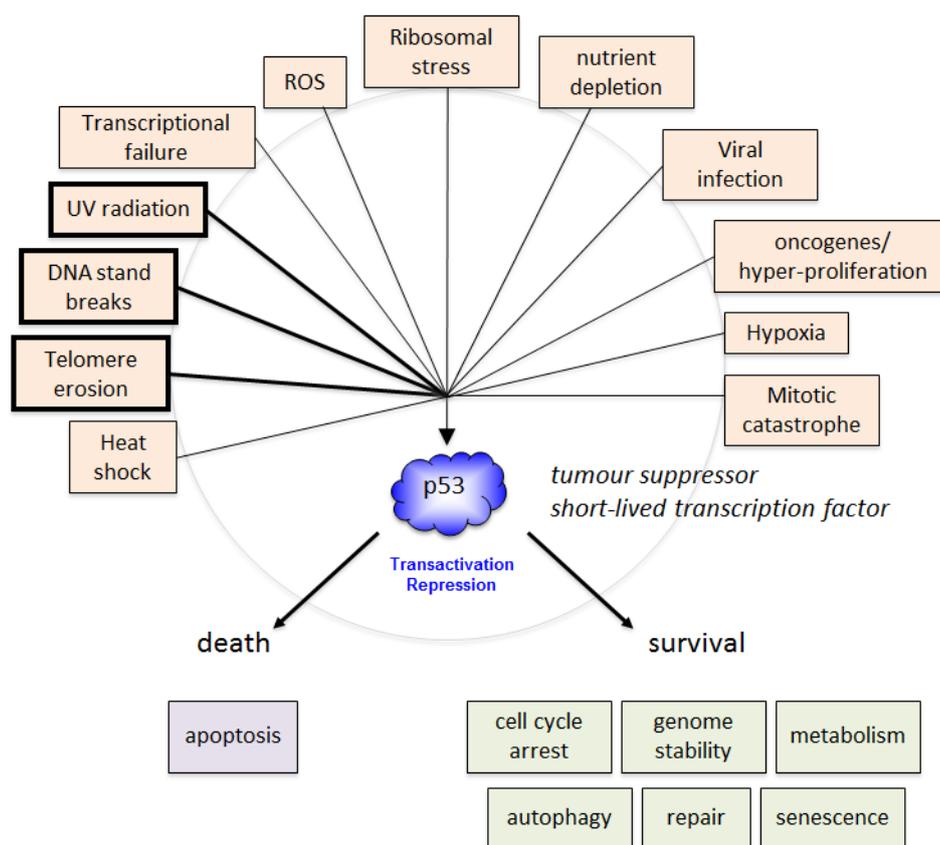
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## Introduction: the p53 protein

The p53 tumour suppressor protein functions principally as a tightly-regulated transcription factor that encompasses both transactivation and repression activities [1-4]. p53 can regulate the expression of hundreds of genes, many of which are involved in mediating or regulating cell growth, division, survival and/or programmed cell death. p53 is a short-lived protein that is regulated mainly through changes in its protein stability [5]. p53 is induced and activated by a range of stress stimuli including activated oncogenes (hyper-proliferation), ribosomal stress and various forms of DNA damage (Fig.

1). Each of these stress stimuli induce p53 essentially by blocking its degradation and lead, in a context-dependent manner, to biological outcomes of growth arrest, senescence or apoptosis, all of which are widely accepted tumour suppression mechanisms that block uncontrolled proliferation of transformed cells. The ability of p53 to regulate the onset of apoptosis is also mediated, in part, at the mitochondrion through its transcription-independent function as a pro-apoptotic BH3-only factor [6].

p53 also regulates genes essential for other cellular processes, often when present at basal, non-induced



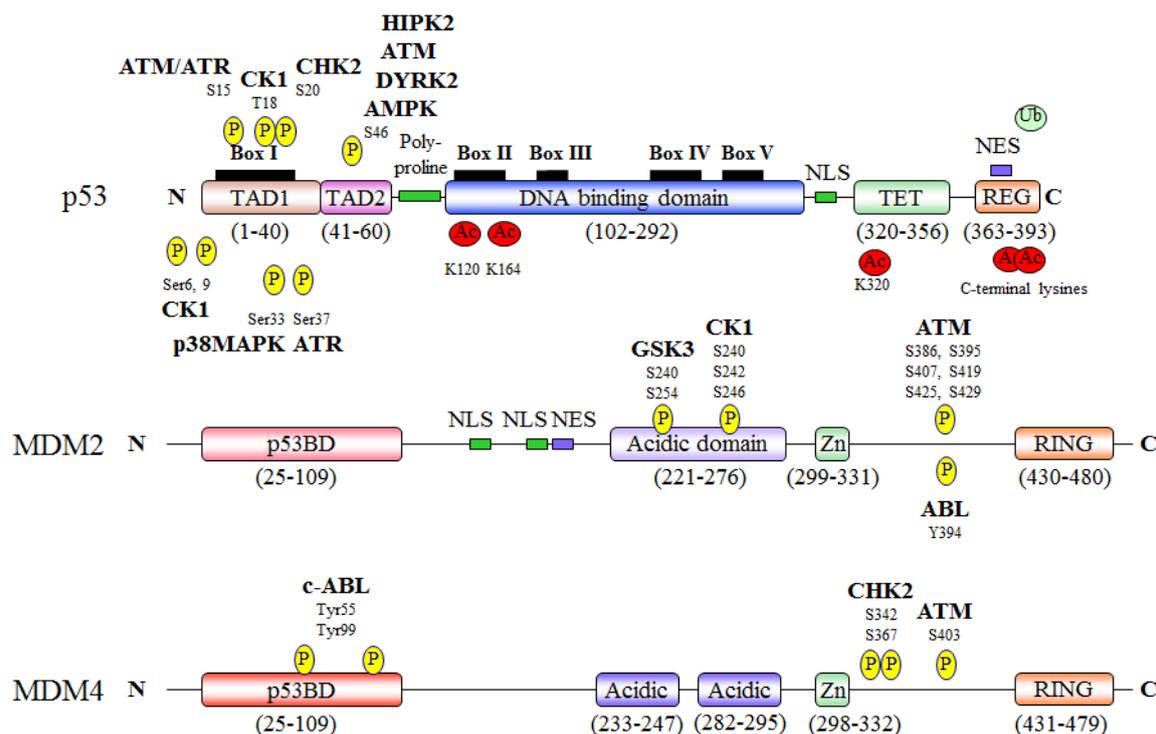
**Figure 1. Stress stimuli that lead to p53 induction.** A wide range of cellular stress stimuli induce p53 leading to coordinated changes in gene expression and various biological outcomes, depending on the cell type and the type, intensity and duration of the activating stress. Those stimuli for which there is unequivocal evidence that phosphorylation events encompass part of their mechanisms of action are indicated by heavy lines.

levels. For example, by regulating expression of leukaemia inhibitory factor (LIF), p53 is able to control implantation and hence fertility [7]. This idea is reflected by the reduced litter sizes frequently seen in p53-null mice. p53 also plays a role in controlling the proliferation and differentiation of stem cells [8] and can function to restrict longevity and promote the ageing process [9, 10]. Through its ability to regulate genes involved in intermediary metabolism and mitochondrial respiration, p53 can reduce flux through the glycolytic and pentose phosphate pathways and stimulate mitochondrial function [4, 11, 12]. This coordinated control favours mitochondrial oxidative phosphorylation as a means of ATP production and minimises the synthesis of substrates needed for growth and cell division. Through targeting of these genes p53 is also able to inhibit the Warburg effect, a cancer-associated phenomenon in which oncogenic processes promote aerobic glycolysis and allow increased flux through the pentose phosphate pathway to provide precursors for cell growth and division. p53 also regulates the IGF-1/mTOR pathways [11], and thus indirectly governs the routes by which proliferation, survival and energy metabolism are controlled, in a manner that is in keeping with its ability to directly control expression of

enzymes involved in intermediary metabolism.

Structurally, p53 comprises several domains that are crucial for mediating its varied functions (Fig. 2). There are two adjoining transactivation domains, termed TAD1 and TAD2 respectively, at the N-terminus. TAD2 overlaps with a proline-rich domain and plays important roles in repression, apoptosis, and the response to gamma-irradiation [13]. The core domain encompasses the site-specific DNA binding function of p53 while the C-terminal region contains sequences involved in nuclear localisation, tetramerisation, non-specific DNA binding and regulation. This regulatory region undergoes a variety of post-translational modifications including ubiquitylation, phosphorylation, methylation and SUMOylation; (these have been reviewed in significant detail elsewhere [14-17]).

p53 is a member of a family that includes p63 and p73, both of which share a high degree of structural similarity with p53 [18]. p63 and p73 are able to activate transcription from many p53 target genes. However they also have important and highly specific functions that are distinct from p53. For example, p63 plays a major role in the development of squamous epithelia while p73 is indispensable for neuronal development. Moreover,



**Figure 2. Modular structure and location of key DNA damage-induced phosphorylation sites in p53, MDM2 and MDM4.** The p53, MDM2 and MDM4 proteins are shown schematically, highlighting important functional domains. In the p53 structure Boxes I-V represent the highly conserved regions of p53 first highlighted by Soussi and May [201]. Those sites of modification that are directly relevant to the DNA damage (strand break) response are shown (yellow ellipses), together with the protein kinases(s) known to phosphorylate them. The sites of acetylation (red circle) and ubiquitylation (green circle) in p53 are also indicated. Comprehensive lists of the post-translational modifications in these proteins are available elsewhere [14, 52, 62, 65, 202, 203].

all three family members cooperate in the regulation of maternal reproduction [7]. p63 and p73 can also contribute to tumour suppression through various mechanisms and can act cooperatively with p53 [18, 19]. Additionally, similar to p63 and p73, p53 comprises a full length protein together with a series of shorter isoforms that arise from alternative splicing and internal starts. These isoforms can interact not only with full length p53, but also with p63 and p73 to regulate their functions [20].

The *TP53* gene (encoding p53) is mutated at high frequency during the development of a wide range of cancers [21, 22]. While this can lead to inactivation of p53 function (as well as the ability to act as a dominant negative inhibitor of wild type p53), many mutant p53 proteins acquire new activities, termed “gain of function”, through which they are able to directly enhance cancer progression and development. These mechanisms include interaction with p63 and p73 to inhibit their transcription programmes; recruitment by transcription factors (e.g. NF-Y) located on relevant promoters leading to increased levels of transcription; direct recruitment to unique sequence-specific elements; and physical interaction with other cancer-related but non-transcriptional proteins [21, 22].

## p53 and tumour suppression

It is well established that p53 provides a critical barrier to the development of many, if not all, cancer types. p53-null mice are susceptible to spontaneous tumour formation, with the elimination of wild type p53 expression in mouse models for various cancers leading to rapid tumour development and death (e.g. see [23-25]). Correspondingly, restoring wild type p53 function in animal models can regress cancer development and significantly extend periods of survival [26-28]. Mouse models have also provided strong evidence that p53-mediated tumour suppression can occur through p53-dependent apoptosis or, alternatively, through p53-induced growth arrest and senescence. They have also established that the relevant importance of these tumour suppressive mechanisms is likely to be cell type-dependent [27]. In contrast, it has been suggested recently that cell cycle arrest, senescence and apoptosis may be dispensable for tumour suppression and that other important cancer-relevant p53 activities such as metabolic regulation or antioxidant function may be required [29].

There is also new evidence indicating that p53-dependent transcription of a large number of its responsive

genes is dispensable for the suppression of some cancers and that only a small number of generally less well-characterised p53-responsive genes (but including BAX) have a crucial involvement in this process [30, 31]. Notably, this subset of important genes does not require the involvement of the p53 TAD1 sub-domain which is a major target for activation by the DNA damage signalling pathways (see below) and which is crucial for the transactivation of many of the “classical” p53-responsive genes involved in growth arrest and apoptosis, including p21, PUMA and NOXA.

The mechanism of induction/activation of p53, and the duration of the response, may be key factors in determining whether p53 tumour suppressor function is activated. For example, developing cancer cells undergo numerous stresses including hypoxia, nutrient limitation, hyper-proliferative signalling (activated oncogenes), and persistent DNA damage. However it is still unresolved what initiating signals and/or pathways arising from such changes are primarily responsible for activating p53 tumour suppressor function. On the one hand, there is good evidence that the DNA damage pathways operate in early developing cancer cells, possibly through oncogene-driven, inappropriate activation of origins of replication, leading to replication fork collapse and strand breaks [32, 33]. DNA damage has therefore been proposed to activate p53 tumour suppressor function. On the other hand, ARF inactivation during lymphomagenesis in an Eμ-Myc model for Burkitt’s lymphoma is sufficient to eliminate p53-dependent tumour suppression [34], strongly suggesting that developing tumours respond principally to hyper-proliferative signals. In support of this idea, two independent studies [35, 36] have shown that the rapid p53-mediated response to DNA damage fails to trigger p53 tumour suppression function. However, restoration of p53 at various time intervals after attenuation of the DNA damage response in these animal models is sufficient to protect against lymphoma development. Importantly, this suppression can be eliminated in an ARF-null background. Consistent with these studies, p53 “super” mice, which have an extra transgenic copy of the intact *trp53* gene, and which show an increased response to genotoxic agents, are unable to suppress tumour formation when crossed into an ARF-null background [37]. Additionally, oncogene-induced senescence and tumour suppression in mice can occur in the absence of a detectable DNA damage response and in an ATM-null background [38]. Taken together, these studies underpin the idea that the ARF pathway is crucial for preventing the development of cancer and question any major role for the acute DNA damage response. However, these analyses have focused on a small number of cancer types and it is possible that there may be variations in the requirement of these pathways depending on tissue- and/or cellular context.

It also remains unclear how other stress stimuli such as hypoxia or nutrient starvation might contribute to mobilising tumour suppression.

## Regulation of p53 by MDM2

p53 is normally kept at low levels through ubiquitylation and proteasomal degradation mediated principally by the RING-finger type E3 ligase MDM2 [39-42]. MDM2 was originally thought to ubiquitylate only six crucial lysine residues in the C-terminus of p53 (K370, K372, K373, K381, K382 and K386) [43] but it is now clear that MDM2 can also target additional lysine residues in p53 *in vivo* [44, 45]. In the presence of high levels of MDM2 p53 becomes poly-ubiquitylated, leading to its degradation. However, p53 can be mono-ubiquitylated by lower levels of MDM2, which can have other consequences such as stimulating p53 nuclear export [46]. MDM2 and p53 operate within a negative feedback loop in which p53 stimulates the expression of MDM2, thus maintaining appropriate levels of its negative regulator [47]. The *MDM2* gene contains two promoters, the stronger of which is dependent upon transactivation by p53. Importantly, the significantly increased levels of MDM2 achieved following the induction of p53 are pivotal in restoring p53 to homeostatic levels following removal of the inducing signal (see below).

The most plausible model for p53 activation is the “anti-repression” model proposed by Gu and colleagues [48]. In this model, p53 is anchored at promoters but kept in a transcriptionally-inactive form through the binding of MDM2 and its relative, MDM4 [48]. (MDM4 (also known as MDMX) is a defective E3 ligase which is highly related to MDM2 and which acts both as a suppressor of p53-mediated transcription [49-52] and as an important stimulatory partner for MDM2 that favours poly-ubiquitylation of p53 [53, 54]). Here, p53 activation occurs through mechanisms that disrupt its interaction with its inhibitory partners. Genes are activated by p53 through subsequent steps such as the recruitment of co-activators (e.g. p300 and CBP), interaction with key subunits of the “mediator” complex, and/or stimulation of RNA polymerase II activity at initiation or elongation steps. In addition to its role in controlling p53 levels, MDM2 can sterically interrupt association of transcriptional proteins with p53 anchored on chromatin and recruit histone deacetylase activity to p53 and neighbouring histone proteins [48]. It is also able to perturb the conformation of the core domain of p53 thereby inhibiting DNA binding by p53 [55]. By promoting mono-ubiquitylation of p53, MDM2 can expose a nuclear export signal on p53, leading to its translocation to the cytoplasm [56, 57]. There is also evidence that MDM2 can inhibit p53 mRNA translation indirectly by mediating the degradation of the ribosomal

protein L26, an activator of p53 mRNA translation [58]. These additional activities complement the ability of MDM2 to down-regulate p53 levels.

MDM2 is the most extensively characterised ubiquitin ligase targeting p53 and is essentially ubiquitous in cells and tissues. It is critical for p53 regulation as exemplified by the lethality of mice lacking MDM2 expression [41, 42]. There are, however, a range of other p53 ubiquitin ligases, discussed in detail elsewhere [59], which also regulate p53 but in a context and possibly cell type-dependent manner. Additionally, p53 operates within several other feedback loops with other proteins, including certain E3 ligases, which regulate its function and/or levels [60]. Likewise, MDM2 interacts with a range of different partner proteins and substrates other than p53, many of which are relevant to cancer [61].

## Regulation of p53 by post-translational modification

In addition to ubiquitylation, the p53 protein is subject to a wide range of other post-translational modifications including multi-site phosphorylation, acetylation, methylation, and SUMOylation. Many of these have been described and discussed in detail elsewhere and will not be revisited here [5, 14-16, 62-65] (Fig. 2).

### A key role for p53 acetylation

Acetylation of lysine residues is considered to be fundamentally important for activating p53 function [66] and there is a large body of evidence describing how the DNA damage pathways can induce p53 acetylation either by directly activating specific acetyltransferases or by stimulating their recruitment by phosphorylating p53 (see below). In the C-terminus of p53 there are six lysines, K370, K372, K373, K381, K382 and K386, which are acetylated by the highly related histone acetyltransferases p300 (a.k.a. KAT3B) and CBP (KAT3A). These same lysine residues are targeted by MDM2 for ubiquitylation. Given that acetylation and ubiquitylation of the same residue is mutually exclusive, one of the key outcomes of acetylation is to block ubiquitylation, thereby serving as a mechanism that opposes the down-regulation of p53 by MDM2 [67]. Acetylation of these residues inhibits p53 export from the nucleus and subsequent degradation. Acetylation of p53 also opposes the recruitment of MDM2 and MDM4 to p53 to form repressive complexes on promoters, and mediates the recruitment of promoter-specific transcription factors leading to activation of transcription [66].

In addition to the six C-terminal lysines, p53 is acetylated at three other important regulatory sites. Acetylation of K320 in the tetramerisation domain is

mediated by PCAF [68] and favours survival over cell death by promoting p53-mediated activation of cell cycle arrest genes [69]. Supporting such a role, a mouse model expressing p53 bearing a lysine to arginine substitution at residue 317 (equivalent to human 320) shows enhanced p53-dependent apoptosis following irradiation [70].

In the core (site-specific DNA binding) domain, acetylation of K120 by TIP60 (KAT5)/hMOF (MYST1/KAT8) occurs rapidly after DNA damage. This modification is considered indispensable for the activation of p53 target genes encoding apoptosis-associated proteins, but thought to have little influence on the expression of genes encoding proteins required for cell cycle arrest [71, 72]. K120 acetylation has also been proposed to contribute to the transcription-independent apoptotic function of p53, while K164, also in the core domain, is a substrate for p300 and CBP and is required for the activation of most p53 target genes [66].

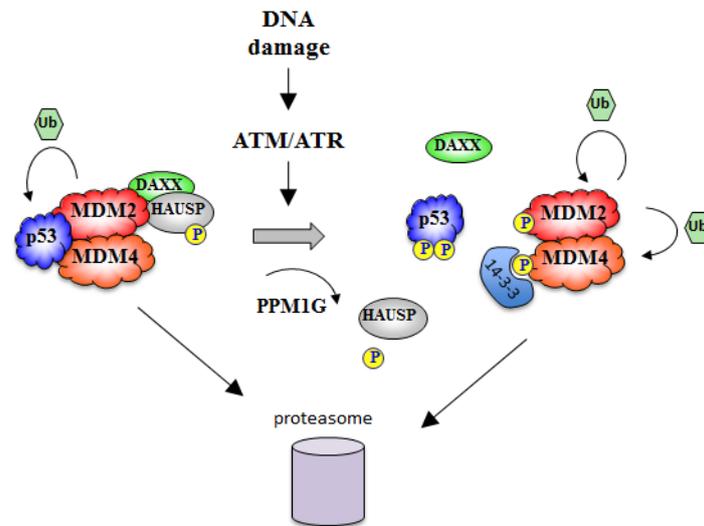
The role of these lysines *in vivo* has been investigated using knock-in mouse models in which either six C-terminal lysines (K367, K369, K370, K378, K379, and K383, equivalent to human K370, K372, K373, K381, K382 and K386 respectively), or these six C-terminal lysines plus K384 (murine specific), are substituted by arginine (p53<sup>6KR</sup> and p53<sup>7KR</sup> respectively). In both cases, however, the mutant mice showed only mild phenotypes and generally showed no major differences in growth arrest, apoptosis or tumour suppression [44, 45], suggesting that modification of these residues might be compensated by other sites, especially given that p53<sup>6KR</sup> and p53<sup>7KR</sup> can be ubiquitylated on other lysines. However in cultured human cells, p53<sup>8KR</sup> (in which the six C-terminal lysines [human p53] and the core domain lysines K120 and K164 are collectively substituted by arginine) is unable to stimulate expression of apoptosis- or growth arrest-related genes. p53<sup>8KR</sup> also fails to induce growth arrest or apoptosis, yet is still able to mediate expression of MDM2 [66]. These findings have been interpreted to suggest that non-acetylated p53 can maintain the negative feedback loop but that acetylation is required to mediate p53 biological function. It will be important to confirm the importance of these additional lysines *in vivo* using mouse models, either individually or in combination with the C-terminal substitutions. Moreover, given that these are lysine/arginine substitutions, they could also be interpreted as mimicking non-ubiquitylated or non-methylated lysines so there should be a degree of latitude in interpretation of these models.

### p53 induction and activation

The main event in the induction of p53 is the uncoupling of p53 from degradation, mediated by MDM2 [5]. This is the focal point of each initiating stimulus and is achieved by

various mechanisms depending upon the inducing signal [5]. p53 is also restrained by other regulators, including MDM4 (above) which act in concert with MDM2 and which may also be targeted during p53 induction (Fig. 3). Importantly, the turnover of MDM4 and MDM2 itself is mediated by MDM2 [73-76] (although there is evidence that other factors can regulate MDM2 turnover such as PCAF [77]). Additionally, p53 turnover involves the actions of the ubiquitin protease HAUSP (also known as

USP7) and the adaptor protein, DAXX [78-84]. HAUSP is able to de-ubiquitylate both MDM2 and p53, each of which competes for the same binding site on HAUSP [85]. Under normal, unstressed conditions DAXX acts as an adaptor that interacts simultaneously with HAUSP and MDM2 and directs the HAUSP ubiquitin protease activity towards MDM2 and MDM4 [82] thereby minimising MDM2 auto-ubiquitylation and promoting p53 ubiquitylation and turnover (Fig. 3).



**Figure 3. Mechanism of p53 activation in response to DNA damage.** p53 is shown together with its main regulators as a complex before activation (left) and following DNA damage-induced phosphorylation and dissociation (right). The mechanism is detailed in the text.

### Induction of p53 in response to activated oncogenes

Activated oncogenes (such as Ras, Myc, E2F-1, beta-catenin) use various mechanisms to drive up the levels of ARF, an inhibitor of MDM2 which is encoded by the gene *CDKN2A* and overlaps with the *INK4A* locus. These mechanisms include stimulation of ARF transcription, inhibition of ARF degradation and segregation of ARF from its targeting ubiquitin ligase, ULF (reviewed in [5]). ARF binds to the central acidic domain of MDM2 and blocks its ubiquitin ligase function. It also accumulates in the nucleolus where it can sequester MDM2, thereby physically segregating MDM2 from nucleoplasmic p53.

### Induction of p53 in response to ribosomal stress

A different mechanism of p53 induction is employed in response to ribosomal stress (also known as nucleolar stress), which arises when the highly coordinated process of ribosome synthesis and assembly is disrupted [5, 86]. This may occur through DNA damage within ribosomal genes and/or through restricted rDNA expression or rRNA processing, giving rise to an excess of ribosomal proteins over and above the amount required for nascent ribosome assembly. In this case, specific ribosomal proteins (namely L5, L11, L23, S3, S7, S14 and S27) can

interact directly with MDM2 within overlapping regions in its central acidic and/or Zn finger regions. The outcome of this binding is the inhibition of MDM2 ubiquitin ligase function leading to p53 accumulation. Mechanistically, this may involve steric hindrance of association of the RING domain with p53, or a reduction in MDM2 flexibility that prevents it from adopting appropriate conformational changes. Additionally, L26 can stimulate p53 translation by binding to the 5' UTR of p53 mRNA [58]. Importantly, Myc can stimulate ribosomal protein translation [87] and may therefore contribute to p53 induction by this route. Consistent with this idea, mice harbouring a cysteine to phenylalanine mutation in the zinc finger domain of MDM2 which mediates L5 and L11 binding, not only fail to respond to ribosomal stress, but show significantly accelerated Eμ-Myc-induced lymphomagenesis, indicating an important contribution of this pathway to p53-mediated tumour suppression and a degree of cross-talk between the ribosomal protein- and ARF-dependent mechanisms of induction [88]. Notably, however, the same amino acid substitution has no effect on the development of prostate cancer development in a mouse model, suggesting that the role of this pathway in cancer prevention is context-specific [89].

### Induction of p53 by MDM2-targeted drugs

The development of drugs which induce p53 offer significant insight into the mechanisms of activation and their downstream effects. Perhaps the best characterised of these drugs is Nutlin-3a (hereafter Nutlin) which competes with the binding of p53 to the N-terminus of MDM2 [90, 91]. The treatment of cells with Nutlin is sufficient not only to induce high levels of p53 protein but to stimulate a robust activation of p53 in terms of its downstream gene expression and biological outcomes. The major effect of Nutlin is simply to inhibit MDM2 and its effects are in accordance with the notion that the uncoupling of p53 from inhibition by MDM2 is sufficient for induction and activation [5].

Other mechanisms can influence the potency of these pathways. For example, survival signalling and/or oncogenic signalling via activated AKT can stimulate MDM2 activity and increase the threshold needed to induce a p53 response [92, 93]. Similarly, increased glucocorticoid levels (arising, for example, through psychological stress) can activate MDM2 via serum- and glucocorticoid-activated protein kinase (SGK1) and increase cancer susceptibility [94]. Additionally, changes in levels of *MDM2* expression in cells, such as those observed in individuals with the single nucleotide polymorphism SNP309, can also significantly affect p53 responsiveness and are associated with increased risk for certain cancers [95].

### The DNA damage pathways: induction, activation and the importance of phosphorylation

The induction and activation of p53 in response to DNA damage is orchestrated by the ATM and ATR protein kinases which are activated by double- and single-strand breaks respectively. A major part of this response involves the coordination and integration of a number of signalling pathways leading to changes in the post-translational status of p53 itself and several of its direct or indirect regulators [5, 14, 15, 52, 62-65] (Fig. 3). The outcome is the uncoupling of p53 from degradation by MDM2 followed by the recruitment of key transcription factors, leading to chromatin remodelling and transcriptional activation. Phosphorylation events on p53 underpin uncoupling from MDM2 and are likely to play significant roles in mediating interaction with other transcriptional components.

#### Phosphorylation of the N-terminus of p53 inhibits MDM2 association and stimulates interaction with transcription factors

In response to double strand breaks, the activation and phosphorylation of p53 are rapid events that occur

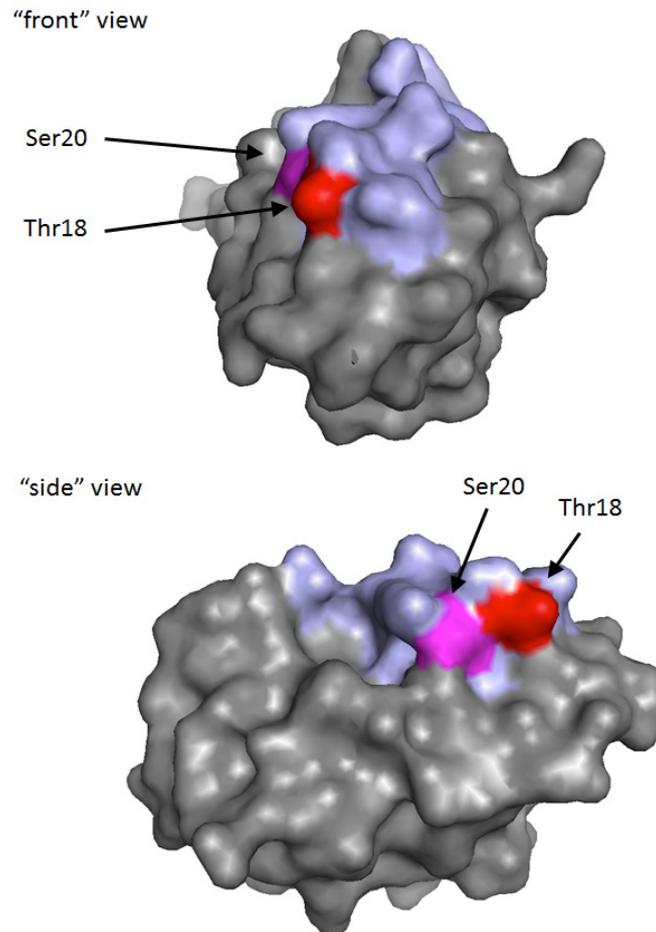
within the first 30 minutes following the stimulus. ATM activation can be transient (lasting only a few hours) and is succeeded, in an overlapping manner, by the activation of ATR, possibly through the generation of single stranded stretches of DNA that are generated by the repair responses. ATM and ATR both phosphorylate Serine 15 in p53 (Fig. 2): thus, the consecutive activation of these two protein kinases provides a continuity of p53 phosphorylation that endures for several hours after the initial stimulus.

Phosphorylation of Ser15 is considered to be an initiating and nucleating event in p53 activation [96] (Fig. 2). Following its modification, protein kinase CK1 can sequentially phosphorylate Thr18 using the phosphorylated Ser15 as a recognition determinant. Peptide-based kinetics experiments, structural studies and protein interaction analyses indicate that phosphorylated Thr18 inhibits p53/MDM2 association and can therefore contribute to uncoupling p53 from degradation [97-103]. Mechanistically, this is thought to occur through electrostatic repulsion of several nearby acidic and aromatic residues at the surface of the MDM2 domain [104, 105] (Fig. 4). Importantly, Ser15 phosphorylation masks a nuclear export signal and therefore contributes to retaining p53 within the nucleus [106]. ATM also phosphorylates and activates the CHK2 protein kinase which, in turn, can phosphorylate Ser20, along with several other residues in p53. Ser20 is also a substrate for other protein kinases that can regulate p53 function under different circumstances/conditions [62, 107]. Ser20 phosphorylation contributes to uncoupling the p53/MDM2 interaction, especially in combination with Ser15 and Thr18 phosphorylation [97, 101, 103, 108, 109]. Interestingly, a mutant p53 in which Ser20 is substituted by alanine is acutely sensitive to degradation by MDM2 [108], underpinning the contribution of this residue as a key regulator of p53 stability. The modification of these residues in response to DNA damage can therefore make a significant contribution to uncoupling the p53/MDM2 interaction.

Other residues in the TAD1 region of p53 are also targeted for DNA damage-induced phosphorylation, including serines 6, 9, 33 and 37 [110] with phosphorylation of Ser9 and Ser33 showing a dependence on ATM [96]. Modification of these residues is not thought to significantly affect the interaction of p53 with MDM2 [101, 103].

#### Multiple phosphorylation events regulate transcription factor/co-activator recruitment and may act cooperatively as a “rheostat” for stimulating p53 activity

In addition to blocking the interaction with MDM2, phosphorylation of individual residues in the N-terminus



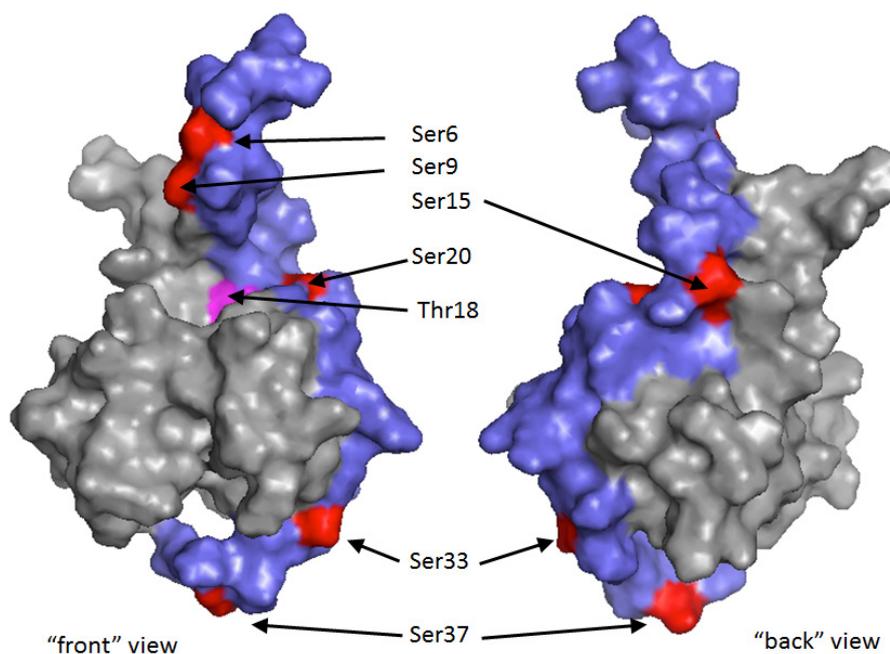
**Figure 4. Location of key phosphorylation sites in the N-terminus of p53 within the context of MDM2 association.** The X-ray diffraction-based structure of an N-terminal peptide of human p53 (pale blue) in complex with the N-terminal domain of MDM2 (grey) is shown. The figure depicts two views: termed "front" and, following rotation of approximately 90° around the vertical plane, "side". The positions of the threonine 18 (red) and serine 20 (magenta) phosphorylation sites are shown. The data are taken from coordinates placed in the NCBI database (accession number: 1YCR).

(TAD1) of p53 stimulates, to various degrees, interaction with p53 binding sites in the p300 and CBP transcriptional co-activator proteins (reviewed in detail previously in [62, 65]). Again, Thr18 phosphorylation has a central role but in this case it functions by mediating tight contact with p300/CBP. Moreover, biophysical analyses measuring the interaction of p53 representative peptides with p300 indicate that di- or multiple-phosphorylation events can act cooperatively, leading to stimulation of the interaction by as much as 80-fold. Analysis of the TAD1 domain of p53 in association with the TAZ2 domain of p300 provides an explanation as to how this may occur. (Note that p300 contains 4 independent but strikingly similar domains termed TAZ1, KIX, TAZ2 and IBID respectively. In the "wrap-around" model proposed by Fersht and colleagues [111], this allows one monomer of p300 to interact simultaneously with each of the subunits in tetrameric p53.) In the context of association with p300, Ser15 and Thr18 lie at the p53/p300 interface (details given in: [99]). The model predicts that phosphorylation of these residues will strengthen contact through increased electrostatic

bond formation with critical residues in p300.

Additional phosphorylation events in the TAD1 region of p53 (serines 6, 9, 33 and 37: mentioned above) may have an important role. Combinations of the various TAD1 phosphorylation events have been demonstrated to act cooperatively in stimulating p300/CBP binding in a graded or incremental manner [100, 101, 103, 112, 113]. They may thus provide a mechanism by which p53 activity can be fine-tuned. The structure of the N-terminus of p53 in association with the p300 TAZ2 domain [99] reveals the close proximity of these phosphorylation sites to the p300 surface where their modification status may influence interaction with key residues in p300 (Fig. 5).

Taken together, the above studies establish two important principles in regulating p53 induction and activation: firstly, that phosphorylation of these key N-terminal sites acts as a *switch* in which rapid uncoupling of MDM2 and recruitment of key transcription factors can occur; secondly that cooperation between the different phosphorylation sites may act as a rheostat to permit fine-tuning of the association between p53 and p300/CBP.



**Figure 5. Location of key phosphorylation sites in the N-terminus of p53 within the context of p53/p300 association.** The NMR-based structure of the N-terminus of human p53 (blue) in complex with the Taz2 domain of human p300 (grey) is shown (99). The figure depicts two views: termed "front" and, following rotation of approximately 180° around the vertical plane, "back". The positions of serine (red) and threonine (magenta) phosphorylation sites are shown. The data are taken from coordinates placed in the NCBI database (accession number: 2K8F).

### Phosphorylations of other key sites in p53 have a major impact on the response to DNA damage and mediate specific interactions with different transcriptional proteins

Other sites in p53 are modified in response to DNA damage, in some cases sequentially depending on events at the N-terminus. Of particular interest, Ser46 in the TAD2 domain is phosphorylated in a manner that may be dependent, directly or indirectly, upon activation of the ATM pathway [96]. Phosphorylation of this residue has been reported to be mediated by various protein kinases, including homeo-domain interacting protein kinase-2 (HIPK2) [114, 115], dual specificity tyrosine-phosphorylation-regulated kinase-2 (DYRK2) [116], protein kinase C-delta [117], AMP-activated kinase-alpha (AMPK-alpha) [118] and ATM itself [119]. Phosphorylation of Ser46 is induced only by relatively high levels of DNA damage stimuli and is proposed to be involved in promoting apoptosis through selective expression of genes such as p53AIP1 [120] and PTEN [121, 122]. Indeed, p53 phosphorylated at Ser46 is preferentially found on the chromatin of apoptosis-related target genes [123]. Thr55, also located in TAD2, appears to be constitutively phosphorylated under homeostatic conditions by protein kinase activity associated with transcription factor TAF1. This modification promotes p53 turnover and cell cycle progression, as well as interaction with the nuclear export factor CRM1, leading to p53 nuclear export [124, 125]. Interestingly, it is

dephosphorylated in response to DNA damage by PP2A [126]. The association of TAD2 of p53 with individual domains of p300/CBP occurs at a different binding interface from the TAD1-interacting region. While phosphorylation of TAD1 greatly influences association with p300/CBP (above), the binding of TAD2 to subdomains of p300/CBP domain is only marginally affected by phosphorylation [100, 101, 103, 112, 113]. In contrast, phosphorylation of p53 at Ser46 and Thr55 increases its affinity for the p62 subunit of TFIIH [127, 128]. These findings underscore the principle that phosphorylation of different sites in different domains can mediate very specific interactions with different components of the transcriptional machinery.

Several other well-characterised phosphorylation sites in p53 can contribute to the DNA damage response [14, 62]. Phosphorylation of Ser6 and Ser9 by CK1 mediates interaction of p53 with Smad proteins and is important for the contribution of p53 to transforming growth factor beta signalling (TGF-beta) [129, 130]: Ser9 is induced in response to DNA damage in an ATM-dependent manner [96, 131]. Ser33 is a target of several kinases [14] and, in particular, is modified by p38MAPK in response to UV [132]. Ser33 phosphorylation also occurs following induction of ARF [133]. Ser37 is a target of ATR and is modified following DNA damage leading to uncoupling of p53 from replication protein A (RPA) [134]. Phosphorylation of Thr81 in the proline domain allows recruitment of the PIN1 peptidyl isomerase

which is thought to modify p53 conformation and generate a binding site for the CHK2 protein kinase, leading to subsequent phosphorylation of Ser20 [135]. Ser155 is phosphorylated by the COP9-signalosome (CSN) and targets p53 for degradation [136]. However, the fate of this modification following DNA damage is unclear. Phosphorylation events on Ser215 and Ser315 by Aurora-A (STK15) have been reported to block site-specific DNA binding and promote turnover respectively, and to counter apoptosis and cell cycle arrest induced by genotoxic agents [137]. In contrast, others find that Ser315 phosphorylation is induced by UV radiation and stimulates p53 transactivation function and tumour suppressor function [138, 139]. A novel site of radiation-induced phosphorylation, Ser269, was recently identified within the “BoxIV/V” region of p53, a highly conserved region considered to be an essential “ubiquitylation signal” that provides a second point of contact with MDM2 [140, 141]. Phosphorylation of this site is thought to inactivate p53 and may induce a conformational shift characteristic of mutant p53 proteins. Ser376 and Ser378 are constitutively phosphorylated in unstressed cells. DNA damage promotes dephosphorylation of Ser376 thereby generating a consensus binding site for 14-3-3 proteins and leading to increased affinity of p53 for sequence-specific DNA [142]. Ser392 is also reported to be constitutively phosphorylated at a low level in unstressed cells. However this is increased by several p53-targeting stimuli including genotoxic agents [133]. Taken together these studies highlight a wide range of phosphorylation events that are sensitive to DNA damage stimuli.

Subtle or even extensive variations in the extent of phosphorylation and other forms of modification of p53 residues occur depending on the inducing stimulus, which governs the type of DNA damage acquired, and the intensity and duration of the stimulus (e.g. see [131, 143]). The ability of these modifications to influence interactions, govern promoter selectivity, and have a bearing on the biological outcome of inducing p53 are a key contributory part of the “barcode” hypothesis: an epigenetic-like code in which the p53 response is tailored, or fine-tuned, appropriately to match and respond to the nature and intensity of the activating stress [144, 145].

## The role of phosphorylation of p53 regulators in p53 induction by DNA damage

Post-translational events in p53 are part of a broader mechanism of induction and activation that involves simultaneous modification of other p53 regulators including MDM2 and MDM4 (Fig. 2). MDM2 and p53 interact tightly through several points of contact that form the targets of inducing signals. A hydrophobic cleft

in the N-terminus of MDM2 serves as a docking site for three key hydrophobic residues in the N-terminus of p53: F19, W23 and L26. Association of p53 and MDM2 through this high affinity interaction is thought to lead to a conformational shift that stimulates an essential low affinity contact between the central acidic domain of MDM2 and the BoxIV/V region of p53 [146-149]: this so-called “ubiquitylation signal” is indispensable for the subsequent interaction of p53 with the RING domain of MDM2 and its ubiquitylation by recruited E2 ligase. Additionally, MDM2 is considered to be a weak E3 ligase but its ability to modify p53 can be stimulated through contact with a number of proteins including dimerisation with MDM4 through their respective RING fingers [53, 54]. Accordingly, DNA damage signals also lead to modification changes in MDM4 that permit p53 activation and accumulation.

In response to strand breaks, ATM phosphorylates MDM2 at various C-terminal sites. Initially this response was thought to be focused on Ser395 [150] but it is now clear that ATM phosphorylates several other residues (Ser386, -407, -425, -429 and Thr419) which can act in a redundant manner in cultured cells [151]. ATR can also phosphorylate Ser407 [152] and perhaps, like ATM, other residues in this region. Mechanistically, modification of one or more of these sites allosterically blocks the ability of MDM2 to form dimers and/or higher order structures mediated through its RING domain, leading to inhibition of its ability to promote poly-, but not mono-, ubiquitylation of p53. Phosphorylation of MDM2 at these sites leads to stabilisation of p53 and thus shuts down the negative feedback loop. Additionally, these modifications can block the contact with p53 that is mediated by the acidic domain of MDM2, thereby eliminating the ubiquitylation signal. A further activity of MDM2 suppressed by ATM-dependent phosphorylation is that of inducing an inhibitory conformational shift in the p53 DNA binding domain [153]. Phosphorylation of MDM2 by the c-ABL protein kinase, which itself is activated by ATM, occurs at Y394 within this cluster and contributes to inhibition of MDM2 [154].

Confirmation that ATM-mediated phosphorylation of MDM2 plays a critical role in the induction of p53 *in vivo* was provided recently following the generation of Mdm2<sup>S394A/S394A</sup> and Mdm2<sup>S394D/S394D</sup> mice [155, 156]; (murine S394 is equivalent to human S395). While the S394A/S394A mice are born at Mendelian ratios and show basal levels of p53 and Mdm2 that are indistinguishable from wild type mice, they are extremely radio-resistant and fail to induce p53-dependent apoptosis in appropriate tissues following irradiation. p53 induction (stabilisation) by IR and downstream gene expression are weak compared with wild type mice but the responses of cells to the MDM2 inhibitor Nutlin are unaffected, thereby underpinning the

central role for S394 phosphorylation in inducing p53. Notably the mutation does not affect MDM2 stability. Interestingly, S394D/S394D mice are phenotypically similar to wild type mice but show delayed attenuation of the p53 response to IR. These studies strongly support the idea that phosphorylation of Ser394 plays a fundamental and potentially dominant role in mediating the induction of p53 in response to DNA damage.

The acidic domain of MDM2 contains a cluster of residues that are phosphorylated under normal unstressed conditions and, based on mutational analyses, contribute towards the normal turnover of p53 [157-159]. These sites are modified, probably in a sequential manner, through the combined action of mainly CK1 and glycogen synthase kinase-3 beta (GSK3 $\beta$ ) [160-162]. In response to IR, hypo-phosphorylation of this domain occurs, which is again consistent with the paradigm that DNA damage stimuli induce p53, at least in part, through uncoupling p53 from MDM2-mediated degradation [157]. The normally abundant activities of these protein kinases fit with the idea that MDM2 can be modified under normal conditions in a manner that maintains the turnover of p53. Mechanistically, hypo-phosphorylation of the acidic domain is thought to occur through the inactivation of GSK3 $\beta$ . In response to ionising radiation, GSK3 $\beta$  is phosphorylated on Ser9 and inhibited by AKT2 [160] which, in turn, is activated *in vivo* by DNA-PK [163, 164]. It is not known at present whether the ATM-mediated dissociation of the MDM2 acidic domain and the BoxIV/V region of p53 (described above) impinges upon or even cooperates with this mechanism.

MDM4 is a defective E3 ligase that is structurally very similar to MDM2 (Fig. 2). Like, MDM2, MDM4 is also targeted by the DNA damage pathways, leading to changes in its phosphorylation status. In response to DNA damage, Ser342 and Ser367 in MDM4 are phosphorylated by CHK2 while Ser403 is directly phosphorylated by ATM [73, 76]. Ser367 is also a target of UV-irradiation where it is modified by CHK1 [165]. Modification of these residues leads to rapid MDM2 binding and MDM2-dependent ubiquitylation and degradation of MDM4. Mechanistically, these phosphorylation events promote inhibition of MDM4/HAUSP binding leading to increased MDM4 ubiquitylation and degradation [81, 166]. Additionally, phosphorylation of Ser267 generates a recognition determinant for the binding of 14-3-3gamma which subsequently stimulates MDM4 ubiquitylation and degradation, and relieves its ability to cooperate with MDM2 in degrading p53 [165-168]. The role of the Ser-342, -367 and -403 phosphorylation sites in MDM4 in contributing towards DNA damage-dependent induction of p53 have been underpinned physiologically through the generation of mice (Mdmx<sup>S341,367,402A</sup>) in which the modification of these residues is blocked by alanine

substitution [169]. Cells from these mice (MEFs and thymocytes) show significantly reduced increases in p53 levels following gamma-irradiation or NCS treatment, supporting an *in vivo* role in regulating p53 levels. Additionally, the mice are radio-resistant and, although they show no increase in spontaneous tumour formation, they do exhibit increased sensitivity to Myc-induced tumorigenesis.

Similar to MDM2, MDM4 is also phosphorylated by c-ABL in response to DNA damage. The modified residues are Tyr55 and Tyr99, both of which are located in the p53 binding domain. Phosphorylation of Tyr99 impairs p53 binding, thereby facilitating p53 activation [170].

The phosphorylation of an isoform of HAUSP (USP7S) also contributes to the DNA damage-mediated induction of p53 [171]. USP7S is normally phosphorylated at Ser18 by the protein kinase CK2. In response to DNA damage, ATM-dependent activation of the protein phosphatase, PPM1G, leads to de-phosphorylation of Ser18 and destabilisation of USP7S with the consequence that MDM2 becomes destabilised and p53 activated.

Taken together, these various studies indicate that DNA damage gives rise to a highly coordinated and integrated process part of which involves the targeting of several interacting proteins involved in promoting p53 turnover (together with many targets in the DNA repair machinery) through protein phosphorylation mechanisms. These events constitute an integrated response in which p53 induction and activation are tailored in accordance with the type, intensity and duration of the initiating stimulus.

## Attenuation of p53 induction by DNA damage

Once induced, activated p53 mediates the expression of key negative regulators, mainly MDM2 and the WIP1 phosphatase [172, 173]. The role of MDM2 in attenuating the p53 response is well established [60]. Interestingly, its role *in vivo* has been significantly underpinned by two recent studies of mutant p53 expression in mice and in zebrafish, before and after treatment with IR [174, 175]. Mutant p53 is maintained at low levels in normal cells by MDM2 expressed from the p53-independent promoter, one of two promoters in the *MDM2* gene. Upon DNA damage, however, both studies show that the levels of mutant p53, which is unable to induce high levels of MDM2 from the alternative p53-sensitive promoter in the *MDM2* gene, significantly exceed wild type p53 levels, induced under identical conditions, and persist for a much longer period.

The WIP1 (PPM1D) phosphatase is also induced by p53 and underpins this response [172, 173]. Analysis of Wip1-null mice has identified physiologically relevant targets for this phosphatase and revealed a major involvement

in regulating the broader ATM, CHK2 and p38MAPK pathways including p53 regulators and p53 itself [176]. These studies have also underpinned the contribution of these pathways to the regulation of tumorigenesis and ageing. Specifically, WIP1 de-phosphorylates Ser15 in p53 [177] and is thought to reduce phosphorylation of other key p53 residues, including serines 20, 33 and 46, through its down-regulation of the pathways that modify these sites [178]. Crucially, WIP1 de-phosphorylates MDM2 phosphorylated at Ser395 leading to destabilisation of p53 [178]. Deletion of Wip1 in mice limits and/or delays tumorigenesis, while WIP1 expression is amplified and over-expressed in many cancers (reviewed in [176]). These findings underpin its importance in cancer suppression. Notably, elevated WIP1 levels are rarely seen in cancers that harbour mutations in p53 consistent with the idea that inappropriate levels of WIP1 can disable p53 tumour suppressor function.

### Mouse models expressing phosphorylation site mutants of p53 and its regulatory partners: what they tell us

A number of studies have carefully considered the biological relevance of DNA damage-induced phosphorylation events in p53 and its principal regulators, MDM2 and MDM4, *in vivo*, by generating knock-in mice bearing amino acid substitutions at one or more of these key positions (139, 156, 169, 179-190). The information gained from these studies has provided valuable insight into their influence on p53 function, their relative importance, and their potential contributions to the prevention of disease. A summary of this information is given in Table 1. There are several interesting conclusions from these studies.

(1) The p53<sup>S18A/S18A</sup> mouse (murine Ser18 is equivalent to human Ser15) was the first to be generated and is the most consistently and intensively studied [179, 180, 189-191]. Analysis of this mouse established that the presence of this key phosphorylation site is required for specific functions of p53 (apoptosis) but does not affect stabilisation, thereby pinpointing a specific role that is in keeping with the cultured cell data. Importantly, this mouse also showed for the first time that Ser18 makes an important contribution to spontaneous (late-onset) tumour suppression and in the suppression of Myc-induced lymphomagenesis. Continued, long-term study of this model has revealed contributions to ageing, embryonic survival and, curiously, glucose homeostasis and insulin sensitivity, findings in keeping with the growing understanding that p53 is a key regulator of metabolism. Therefore, in addition to its contribution to tumour suppression, phosphorylation may have unanticipated but fundamentally important roles in other key areas of p53

biology.

(2) There is cooperation between different modification sites *in vivo*, reflecting the model described above based on biochemical and cultured cell analyses. This is perhaps best exemplified by the p53<sup>S18,23A/S18/23A</sup> mouse [188] where there is a clear synergism between the two sites in activating p53-dependent apoptosis after DNA damage as compared with the outcome of substituting either site alone.

(3) While the mice collectively demonstrate that phosphorylation is not essential for tumour suppression, they clearly show that phosphorylation contributes, probably in a cell- or tissue-dependent manner, to inhibiting cancer development.

(4) The effects of substituting phosphorylation sites on p53 function in cells of lymphoid lineage are more acute as compared with those in fibroblasts. These differences highlight the possibility that phosphorylation of p53 may play fundamental roles in certain cell types but may be redundant or even irrelevant in others. It could be argued that such differences in cell behaviour could reflect artifactual changes acquired upon isolation of the cells and growth in culture. However, the increased sensitivity to lymphoma development in several of the mice tends to argue against this and may therefore support lineage-dependent behaviour.

(5) Individual phosphorylation sites in p53 may have highly specific and/or cell type-dependent roles *in vivo*. For example, the S389A mice show a striking selectivity in gene expression and in contributing to tumour inhibition [181, 182, 186].

(6) While individual, or combinations of, phosphorylation sites in p53 have subtle and cell specific roles, the targeting of MDM2 via the DNA damage pathways (via Ser394 phosphorylation) has a *major* effect on the ability to induce and activate p53. This dominance of MDM2 fits well with the growing understanding that uncoupling the p53/MDM2 interaction is the key principle underlying p53 activation [5]. Moreover, the finding that 65% of MDM2<sup>S394A/S394A</sup> mice develop spontaneous lymphomas within 24 months (a rate similar to that observed in p53<sup>+/-</sup> mice) suggests a strong link between p53 induction through the ATM/ATR pathways and tumour suppression.

(7) In the growing list of biological functions of p53, its ability to coordinately regulate the levels of key metabolic enzymes has acquired enormous significance, both in a physiological context and in the context of cancer prevention by opposing the Warburg effect (aerobic glycolysis in cancer cells) [11, 192]. The demonstration that S18A mice show defects in glucose metabolism and acquire a degree of insulin insensitivity is therefore very striking [180]. This takes on additional significance when one considers that ATM, once thought

Table 1.

Genotype	Phenotype	Spontaneous tumours	Tumours following carcinogenic induction	p53 activity	p53 stabilisation	Reference
p53 <sup>S18A/S18A</sup>	born at expected frequency	mainly late-onset lymphomas plus some other unusual tumours	susceptible to Myc-induced lymphomagenesis	reduced transactivation of PUMA in thymocytes; C-terminal acetylation defective; reduced apoptosis of thymocytes and splenocytes after IR; no significant changes in gene expression or arrest of MEFs; metabolic stress and defects in glucose homeostasis	unchanged	179, 180, 183, 189, 190
p53 <sup>S23A/S23A</sup>	born at expected frequency; life span slightly reduced	various B cell-lineage tumours (mainly)	not tested	thymocytes, splenic B cells and cerebellar neurones partially resistant to IR-induced apoptosis	no effect in MEFs but reduced in thymocytes	188
p53 <sup>S18,23A/S18,23A</sup>	born at expected frequency; normal ploidy; premature ageing in <i>Xrcc4</i> <sup>-/-</sup> background	mainly thymic lymphomas (develop within 6 months); other tumours in a wide spectrum of tissues	not tested	apoptosis of thymocytes abolished; significantly reduced gene expression in thymocytes; partially reduced gene expression in MEFs; impaired arrest in MEFs; slightly faster proliferation of MEFs	impaired in thymocytes	184
p53 <sup>T21D,S23D/-</sup>	homozygous mutants rarely obtained; mice runted; depletion of adult stem cells in multiple tissues; segmental progeria and death within 6 weeks	No	not tested	constitutive p53 activation	increased	187
p53 <sup>S46A/S46A</sup> (humanised)			not tested	impaired expression of certain genes; partially impaired apoptosis of Ras-expressing MEFs; IR-induced apoptosis of thymocytes abolished	reduced	185
p53 <sup>S312A/S312A</sup>	born at expected frequency; life span normal	No	increased susceptibility to thymic lymphomas after IR	reduced during embryogenesis; reduced transcriptional response	unchanged	139
p53 <sup>S389A/S389A</sup>	viable and normal	No increase compared with wt but slight difference in tumour spectrum	increased susceptibility to UV-induced skin tumours and carcinogen-induced bladder cancers	impaired expression of certain genes after UVC; impaired apoptosis (selective) following UV but not IR or oncogene activation; small decrease in basal expression of many genes	unchanged	181, 182, 186
Mdm2 <sup>S394A/S394A</sup>	born at expected frequency	16X increase in spontaneous tumours: mainly T cell lymphomas	not tested	extremely radio-resistant; fail to induce p53-dependent apoptosis in tissues after IR; fail to induce p53-dependent gene expression	greatly reduced	156
Mdm2 <sup>S394D/S394D</sup>	born at expected frequency	No	not tested	persistently elevated p53 levels and downstream expression after IR	unchanged	156
Mdmx <sup>S341,367,402A/S341,367,402A</sup>	born at expected frequency	No	increased susceptibility to Myc-induced tumorigenesis	radio-resistant; impaired p53-dependent gene expression; impaired Mdmx degradation	impaired	169

solely to mediate signals arising from DNA damage, has now been implicated in areas such as metabolism and insulin sensitivity, oxidative stress, ageing and mitosis [193-197]. This raises the possibility that targeting of p53 and its associated regulators by ATM may extend beyond the boundaries of the DNA damage response and mediate sensitivity to, and surveillance of, other key biological processes.

## Perspectives

The wealth of study on the role of p53 phosphorylation discussed above raises a number of interesting issues for further consideration and exploration.

### What is the role of phosphorylation?

Recent evidence has underpinned the idea that, while DNA damage signals impinging on p53 lead to multiple phosphorylation events on key players including MDM2, MDM4 and p53 itself, the targeting of MDM4 [169] and especially MDM2 [156] provides a means of uncoupling p53 degradation, leading to increased cellular p53 levels. This mechanism fits with the idea that most (if not all) stress stimuli that activate the p53 pathway do so mainly by regulating MDM2 function and have evolved efficient mechanisms to achieve this [5]. Assuming this to be the case, the question then arises as to why the DNA damage pathways should require such multi-site phosphorylation events whereas other pathways (e.g. hyper-proliferation, ribosomal stress) do not seem to need these? One possible answer is that phosphorylation helps implement the “Barcode hypothesis” [144, 145]: in other words it may help tailor the type and intensity of the p53 response in a manner adaptive to the type and intensity of the DNA damage and, possibly, to the needs of the individual cell type. However, while such fine tuning would provide an exquisitely sensitive mechanism of dealing with damage, it raises the question of why other types of stress do not use, or perhaps do not need, such a sensitive system. It may be that the complexity of events involved in detecting damage to, and mediating repair of, DNA necessitate an equally complex surveillance from p53 in order to achieve the most appropriate outcome.

An alternative possibility is that the efficiency of this induction process is improved by the sort of coordinate control that multi-site phosphorylation applies to MDM2, p53 and other proteins involved in regulating p53 levels: e.g. inactivating MDM2 and MDM4 while simultaneously blocking their interaction sites on p53 could ensure the outcome and increase the strength of the induction. Moreover, if induction occurs via the “anti-repression” model [48], where p53 is anchored to promoters and repressed *in situ* by

MDM2 and MDM4, this could be used to selectively or preferentially activate certain genes if it were possible to simultaneously disengage MDM2/MDM4 and regulate the access of the signalling enzymes to these particular promoters. This would fit with any potential contribution of phosphorylation to the “Barcode hypothesis.” A further point to consider is that different *levels* of phosphorylation may be required depending on the stress stimulus; for example many studies have observed low levels of Ser15 phosphorylation when inducing p53 through DNA damage-independent mechanisms (e.g. see [133]).

### What does phosphorylation do on chromatin?

Related to points raised in the previous paragraph is the issue of the role played by multi-site phosphorylation in the context of chromatin where p53 conducts most of its business. It is clear that phosphorylation can block MDM2 interaction and promote recruitment of key transcription factors. However, other (DNA damage-independent) stresses are also able to recruit transcription factors so is there a fundamental requirement for phosphorylation in this process? This seems unlikely given that other stress stimuli do not induce these modifications on p53. However, it may be that DNA damage is a special case: thus, for example, if DNA damage-mediated modification of other transcriptional components occurs, (or, indeed recruitment of different transcription factors), p53 phosphorylation may be required to be compatible with these. Or perhaps selectivity in the *strength* of inducing certain genes is required (i.e. turning them up or down as compared with other stresses as opposed to on or off). Given that small changes in p53 activity can have major impacts on cell fate, the value of subtle levels of regulation cannot be over-emphasised.

### What p53-dependent biological outcomes are actually regulated?

The evidence discussed above, particularly that from the animal models, indicates that phosphorylation of p53 itself is likely to have a bearing on the ability of p53 to mediate tumour suppression (Table 1). However, these data collectively indicate that such a role is contributory as opposed to fundamental and may actually have cell- or tissue-type relevance. If phosphorylation of p53 is not essential for tumour suppression, then what function(s) has it evolved to regulate? One possibility is stem cell status where, for example, Ser315 phosphorylation appears to play an important role in controlling *nanog* expression [198]. Another is mitosis where ATM activation by Aurora-B was shown recently to be required for the spindle checkpoint [196]. p53 is not directly required for this checkpoint but has long been known to mediate growth arrest or apoptosis following failure

of the mitotic checkpoint, aberrant mitosis and re-entry into cycle [199]. The involvement of ATM in mitosis raises the question as to whether p53, an ATM target, can somehow sense the fidelity of mitotic events through its interaction with, and phosphorylation by, ATM. Phosphorylation events may also be crucial in mediating the ability of p53 to govern metabolism (discussed above). For example, there are now strong links between ATM function and metabolism [197]. Moreover, p53<sup>S18A/S18A</sup> mice show metabolic defects, and p53 can regulate the expression of many metabolic enzymes including the sestrins which are pivotal regulators of AMPK signalling and levels of reactive oxygen species (ROS) [180]. Further analysis of the various phosphorylation site mouse models in the context of these and other emerging physiological roles for p53 may prove to be of considerable interest.

### Is there a role for phosphorylation of mutant p53?

Mutant p53 proteins are now known to help drive tumour progression and metastasis. Additionally, it is becoming clear that many of the drugs used in the clinic can activate the cancer-promoting functions of mutant p53 [200]. Given that many of these agents are genotoxic

compounds that are known to stimulate phosphorylation and activation of wild type p53 it follows that they may also lead to phosphorylation of *mutant* p53 proteins, especially since the signalling pathways that mediate these phosphorylation events are often still very active in cancer cells. Moreover, since at least some functions of mutant p53 require an intact N-terminus ([21] and references therein), the phosphorylation of key sites in the context of a mutant p53 may permit interactions with key transcription factors to occur. While such interactions should have little effect on normal p53 target genes, they could sequester important transcription factors needed for the expression of other key genes. They may also stimulate the expression of those genes in whose expression mutant p53 is known to play a significant role [21]. Such a scenario would have serious implications for the treatment of cancer patients, raising the possibility that standard chemotherapeutic treatments could actually stimulate the disease in those patients with appropriate p53 mutations. It might also help to explain why mutations in the *TP53* gene encoding phosphorylation target residues are rarely, if ever, seen in human cancers as the fidelity of such residues may be required to underpin the potency of mutant p53 proteins.

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