p53 N-terminal phosphorylation: a defining layer of complex regulation

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The p53 tumor suppressor is a critical component of the cellular response to stress. As it can inhibit cell growth, p53 is mutated or functionally inactivated in most tumors. A multitude of protein–protein interactions with transcriptional cofactors are central to p53-dependent responses. In its activated state, p53 is extensively modified in both the N- and C-terminal regions of the protein. These modifications, especially phosphorylation of serine and threonine residues in the N-terminal transactivation domain, affect p53 stability and activity by modulating the affinity of protein–protein interactions. Here, we review recent findings from in vitro and in vivo studies on the role of p53 N-terminal phosphorylation. These modifications can either positively or negatively affect p53 and add a second layer of complex regulation to the divergent interactions of the p53 transactivation domain.

Introduction

The p53 tumor suppressor protein is a key component of the cellular stress response. p53 is activated by DNA damage, hypoxia, heat shock and other stresses and, depending on the cellular context and the nature of the stress, regulates the cellular responses of DNA repair, cell cycle arrest, senescence and apoptosis. The stress response enacted by p53 derives primarily from its function as a transcription factor. p53 activates or represses the transcription of a large number of genes, including PUMA, CDKN1A (p21) and MDM2 (1), in part through sequence-specific interaction with DNA. It serves as a critical monitor of genome stability; as such, it is mutated in approximately half of all human tumors (2). Mutant p53 facilitates tumor formation both through dominant-negative inhibition of wild-type p53 and gain-of-function roles (recently reviewed in ref. 3). Furthermore, in the majority of tumors retaining wild-type p53, it may be functionally impaired by misregulation, such as through overexpression of its repressor Mdm2 (4).

p53 is a multi-domain protein (Figure 1). At its N terminus is the transactivation domain (TAD), important for interaction with transcriptional coactivators and corepressors. The TAD is composed of two homologous subdomains, TAD1 (residues 1–40) and TAD2 (residues 41–61), which both contain conserved two homologous subdomains, TAD1 (residues 1–40) and TAD2 (residues 41–61), which both contain conserved residues (5). The TAD is composed of transactivation domain (TAD), important for interaction with tran-

Abbreviations: CBP, CREB-binding protein; HUPKI, human p53 knock-in; IR, ionizing radiation; MEF, mouse embryonic fibroblast; NCBD, nuclear receptor coactivator-binding domain; NMR, nuclear magnetic resonance spectroscopy; REG, regulatory domain; RPA, replication protein A; TAD, transactivation domain.

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protein half-life (Figure 2B) (23,26–28). All three knock-in mutant mice displayed some defect in p53-dependent stress responses. Following exposure to IR, p53-dependent apoptosis in thymocytes from $p53^{S18A/S18A}$ mice was impaired, as was UV-induced G1/S arrest in mouse embryonic fibroblasts (MEFs) (26). Although G1/S arrest following exposure to IR was unaffected in $p53^{S23A/S23A}$ MEFs, apoptosis in thymocytes was moderately reduced (28). In contrast, cells from $p53^{S18A,S23A/S18A,S23A}$ mice were severely compromised in p53-dependent apoptosis (27), with overall levels of apoptosis in γ-irradiated thymocytes similar to those in p53$^{-/-}$ thymocytes. In comparison, knock-in mice containing mutations of residues Leu25 and Thr26 (Leu22 and Thr23 in humans) to glutamine and serine, respectively (p53.L25Q,W26S/L25Q,W26S; Figure 2A), have impaired G1/S cell cycle arrest and very low rates of apoptosis after DNA damage (31–33). These mutations abrogate p53 transactivation by transcriptional coactivators.

The impairment in p53-induced stress responses correlates with specific effects on the induction of p53 target genes. For example, the increased expression of Cdkn1a, Perp, Sfn and Tnf after IR was significantly impaired in p53$^{S18A/S18A}$ thymocytes, whereas Mdm2, Noxa, Bax, Apaf-1 and Wig1 induction was unaffected (26). Decreased histone acetylation at the Cdkn1a promoter was observed in thymocytes from $p53^{S18A/S18A}$ mice compared with wild-type mice, whereas histone acetylation at the Mdm2 promoter was unaffected (26). Furthermore, the REG domain of p53 from p53$^{S18A/S18A}$ MEFs showed less acetylation following exposure to UV than in wild-type MEFs. The induction of p53 target genes in $p53^{S18A,S23A/S18A,S23A}$ thymocytes was more dramatically decreased, with changes similar to those in p53$^{-/-}$ cells after IR (27). For comparison, p53.L25Q,W26S/L25Q,W26S mice were defective in transcriptional activation of most p53 target genes, including Cdkn1a, Noxa and Puma, although Bax expression was unaffected (31–33). The similarity in the phenotypes of posttranslational modification knock-in mice and the p53.L25Q,W26S/L25Q,W26S knock-in mice demonstrates that these modifications are critical in regulating the stability and activity of p53 after stress.

Although born at the expected ratio, knock-in mice containing a single p53 allele with mutation of Thr21 and Ser23 to aspartic acid (p53T21D,S23D−−), which mimics constitutively phosphorylated p53, exhibited premature aging and a significantly reduced life span of only 6 weeks (35). Two copies of the mutated allele resulted in embryonic lethality. Cells from p53T21D,S23D−− mice showed increased p53-dependent transcription and apoptosis in the untreated state as compared with p53$^{-/-}$ cells, but this activity was unaffected by DNA damage and was lower than that observed in p53$^{-/-}$ cells after damage. Thus, although the aspartic acid mutation imperfectly mimics phosphorylation, the results in p53T21D,S23D−− mice are consistent with other knock-in mice in demonstrating the importance of phosphorylation in modulating p53 function.

Recent studies have demonstrated involvement of p53 in regulating cellular metabolism. p53 can promote oxidative phosphorylation, inhibit glycolysis and regulate several mitochondrial and nonmitochondrial genes involved in metabolism (recently reviewed in ref. 36). Consistent with these activities, insulin and fasting blood triglyceride levels were increased in $p53^{S18A/S18A}$ mice (24). Additionally, 24-week-old mutant mice showed a modest increase in body weight as compared with wild-type mice and exhibited glucose intolerance and insulin resistance. Crossing p53$^{S18A/S18A}$ mice with Atm$^{−−}$ mice resulted in reduced embryonic viability and decreased weight of surviving offspring as compared with Atm$^{−−}$ mice (25). Finally, tests of motor coordination demonstrated a gender-specific effect of the Ser18 mutation. These studies demonstrate the importance of p53 phosphorylation not only for its DNA damage response functions but also for its role in regulating energy pathways in the cell.

Concordant with the functional defects in p53 signaling, p53$^{S18A/S18A}$, p53$^{S23A/S23A}$ and p53$^{S18A,S23A/S18A,S23A}$ knock-in mice were prone to development of spontaneous tumors at 1–2 years of age (23,27,28). Unlike p53$^{-/-}$ mice, which develop thymic lymphomas, the knock-in mice predominantly developed B-cell lymphomas (23,26,37). Thus, the loss of p53 phosphorylation has specific functional consequences that overlap with, but do not fully recapitulate, protein loss.

Mutation of Ser46 to alanine was generated in the HUPKI mouse model, as this residue is not conserved in mice (Figure 2A). Since the signaling pathway that leads to its phosphorylation is conserved, the mouse model adds important understanding regarding this site of modification in a physiological setting. Unlike the TAD knock-in mice, MEFs and thymocytes from p53S46A/S46A HUPKI mice showed decreased p53 levels after exposure to UV and IR, respectively, as compared with the corresponding wild-type HUPKI mouse cells (30). This result is interesting, as Mdm2 binds exclusively within TAD1. The decreased stabilization of p53S46A/S46A in thymocytes was accompanied by a moderate decrease in transactivation of p53 target genes and a modest reduction in p53-dependent apoptosis.

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**Fig. 1.** Domain structure of p53 showing known sites of posttranslational modification. Documented sites of p53 posttranslational modification are shown; known modifying (black arrow) or unmodifying (red arrow) enzyme(s) are indicated above the modification. Circle: serine (yellow) or threonine (orange) phosphorylation; hexagon: ubiquitin or ubiquitin-like modification; square: acetylation; oval: N-acetylglucosamine; octagon: poly ADP-ribosylation; pentagon: arginine (gray) or lysine mono- (lavender) or di- (violet) methylation; star: nitration.
In thymocytes from p53 S18A/S18A mice compared with wild-type mice, apoptosis in thymocytes was moderately reduced (28). In contrast, Cdkn1a knock-in mice were defective in transactivation of most p53 target genes, including Noxa and Bax expression was unaffected, Tnf expression was unaffected, and mitochondrial genes involved in metabolism (recently reviewed). p53 can promote oxidative phosphorylation, inhibit glycolysis and regulate several mitochondrial and non-mitochondrial genes involved in metabolism. p53 can promote oxidative phosphorylation, inhibit glycolysis and regulate several mitochondrial and non-mitochondrial metabolism.

Structural features of p53 TAD complexes and effects of phosphorylation

The p53 TAD is phosphorylated by a number of activated kinases and is critical for the many protein–protein interactions that either modulate the stability and subcellular localization of p53 or effect its function as a transcription factor. When unbound, the TAD is unstructured (38), but it adopts a helical conformation upon complex formation. Intrinsic disorder is a common feature of ‘hub’ proteins such as p53 that interact with a large number of binding partners (39,40). Rather than forming an extended random coil, recent studies indicate that the free TAD is in a partially compact collapsed state, with transient elements of helical secondary structure (41). The partially folded nascent helical structures lower the energetic barrier to induced-fit binding but concomitantly enable promiscuous binding (42).

Range of bound structures. To date, structures of p53 TAD in complex with six different partner proteins have been reported. In all cases, the complexes contain portions of the TAD bound to an isolated domain of the interacting protein. As depicted in Figure 3A, the structures exhibit considerable diversity: one or both subdomains can bind individually or the two can bind simultaneously. The helix lengths and locations also vary among the complexes (Figure 3A), although in all cases, a Φ-X-Φ-Φ motif is included. These helices are amphipathic, leading to a mixture of hydrophobic and electrostatic interactions with the binding proteins.

In the first structure reported was the complex of p53 with the N-terminal domain of human Mdm2 (Figure 4A) (43). The minimal Mdm2-binding region resides fully within TAD1, which forms a helix encompassing residues 19–25 (Figure 3A). In cells, complex formation results in ubiquitination of the p53 REG domain by the C-terminal E3 ligase domain of Mdm2 (Figure 1), leading to nuclear export and degradation of p53. Since Mdm2 is a transcriptional target of p53, these two proteins form a negative feedback loop that controls p53 levels in the absence of stress and during the return to homeostasis following stress. In the complex, three highly conserved hydrophobic residues (Phe19, Trp23 and Leu26) align along one face of the TAD1 helix and are packed deeply in a hydrophobic cleft of Mdm2 (43). This results in a relatively strong complex (Kd ~ 100 nM) stabilized primarily by the hydrophobic effect. Similar structures have been determined for the interaction of TAD1 with MdmX, a homolog of Mdm2 that also negatively regulates p53 (44,45).

In contrast, the interaction of p53 with CBP or p300, two homologous histone acetyltransferases, facilitates its transcriptional activator activity. By binding the TAD, these proteins are recruited to p53 binding sites in chromatin near the promoters of target genes, resulting in modification of proximal histones and chromatin unwinding (46–48). Additionally, CBP and p300 acetylate lysine residues in the REG domain, which further stabilizes and activates p53 (Figure 1). Inhibition of binding by competitor proteins or downregulation of CBP/p300 by small interfering RNA compresses p53-mediated transcriptional activation and reduces local histone acetylation at promoters of p53 target genes (47,49–51). Furthermore, catalytically inactive deletion mutants of p300 dominantly inhibit p53-dependent apoptosis and G1 arrest (52,53).

CBP and p300 are composed of eight distinct domains, five of which have been shown to interact with the p53 TAD in vitro: Taz1(CH1), IHD, KIX, Taz2(CH3) and the nuclear receptor coactivator-binding domain (NCBD, also referred to as IB1D) (52,54–58). The affinities of p53 for these isolated domains range from 0.02 to 10 μM (59–62).

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**Fig. 2.** Phenotypes of p53 knock-in mice. (A) Sequence of human and murine p53, showing sites of mutation in p53 knock-in mouse models. (B) Summary of phenotypic observations for various knock-in mice.
It should be noted that the binding affinities vary between studies, probably because of differences in the specific method used to measure the affinity and buffer conditions. We and others have shown that several of these domains interact with both TAD1 and TAD2 individually (61,63,64). For interaction of isolated TAD1 or TAD2 with the Taz2 domain of p300, we found that both subdomains bound with equal affinity to the same face of Taz2 (63,65). In contrast, the NCBD and KIX domains each have two distinct binding sites, allowing for the simultaneous binding of TAD1 and TAD2 in the context of the intact TAD (66).

To date, the structures of only two p53-CBP/p300 complexes have been determined: TAD1 with p300 Taz2 and a contiguous TAD1/2-containing peptide with CBP NCBD (Figure 3A). The former complex differs considerably from the TAD1-Mdm2 complex (Figure 4) (65). First, TAD1 forms a longer helix (residues 15–27 versus 19–25; Figure 3A). Second, the side chains of Phe19, Trp23 and Leu26, which are buried in the TAD1-Mdm2 complex, are significantly exposed to solvent in the TAD1-Taz2 complex (especially Trp23 and Leu26). For the TAD1-Taz2 complex, the major stabilizing hydrophobic interactions come from burial of p53 Leu22 and Leu25; individual alanine substitutions of these residues led to complete loss of binding (65). The TAD1-Taz2 complex is further stabilized by electrostatic interactions, including salt bridges involving p53 Glu11 and Glu17 and hydrogen bonds with Ser15, Thr18 and Asp21.

In the TAD-NCBD complex, the p53 TAD wraps around the CBP NCBD, allowing the helical portions of TAD1 and TAD2 to bind simultaneously at proximal sites (Figure 4C) (67). The non-helical portions of the TAD peptide are less well defined, as demonstrated by the range of conformations in the Nuclear Magnetic Resonance spectroscopy (NMR) models (67). In addition to Phe19, Trp23 and Leu26 of TAD1, Ile50, Trp53 and Phe54 of TAD2 are buried with minimal solvent exposure in a non-polar pocket of the NCBD. While these hydrophobic interactions provide the major stabilization of the complex, some salt bridges and hydrogen bonds may form at the periphery.

Binding of p53 TAD2 to the pleckstrin homology (PH) domain of the p62 subunit of TFBH (and its yeast homolog Tfb1) facilitates p53-dependent activation of transcription (68). In the structure of TAD2 bound to Tfb1, the three hydrophobic motif residues (Ile50, Trp53 and Phe54) each bind in a non-polar pocket of the PH domain (Figure 4D). Consequently, as with both the Taz2 and NCBD complexes, binding is primarily stabilized by hydrophobic interactions and supplemented by salt bridges, hydrogen bonds and cation/ aromatic interactions.

The fifth complex depicted in Figure 3A is that of TAD2 bound to the N-terminal domain of the largest subunit of the heterotrimeric replication protein A (RPA) complex, RPA70N (69). This is an example of a separate class of p53-interacting proteins that recognize the p53 TAD as a single-stranded DNA mimic. Other members of this class include the respective C-terminal DNA-binding domains of positive cofactor 4 (PC4), BRCA2, and the human mitochondrial single-stranded DNA binding protein (HmtSSB). In complex with RPA70N or BRCA2, TAD2 binds to the oligonucleotide/oligosaccharide-binding folds of the cofactors, directly competing with single-stranded DNA (69,70), whereas both subdomains bind to HmtSSB (71). Binding of PC4 to the TAD, DNA-binding domain and REG domains of p53 promotes the transcriptional activation of p53 by increasing binding to its DNA response element (72,73). In contrast, interactions with RPA70N and BRCA2 repress the transcriptional activity of p53, possibly by preventing contact with other cofactors. As shown in Figure 4E, the hydrophobic consensus residues of TAD2 bind as a cluster in a hydrophobic pocket of RPA70N. A second region of helical structure in TAD2 formed by residues 36–44 also contributes to binding. For this class of binding proteins, electrostatic interactions play a significant role in complex stabilization. While mutation of hydrophobic residues in p53 TAD reduced its affinity for BRCA2 and PC4 by 5- to 10-fold, substitution of the acidic residues completely eliminated complex formation (70,73). This reflects a unique type of interaction with this class of cofactors in which acidic residues in TAD compete with negatively charged phosphate groups of single-stranded DNA.

Effects of phosphorylation. The importance of TAD phosphorylation in the regulation of p53 function has led to numerous in vitro studies examining the effects of p53 phosphorylations on interactions with its binding partners. These typically utilize biophysical techniques such as fluorescence depolarization, isothermal titration calorimetry or NMR chemical shift perturbation studies. Due to the –2e formal charge under physiological conditions, phosphorylation can be thought of as adding an electrostatic-based component to the binding energy. For the interaction with Mdm2, which is primarily stabilized by the hydrophobic effect, phosphorylation prevents complex formation. In
contrast, TAD phosphorylation enhances binding to CBP/p300 and p62. Thus, phosphorylation couples relief of negative regulation with enhancement of transcriptional activation. To this end, the structures of p53 complexes have facilitated understanding the detailed steric and physiochemical bases of the effects of phosphorylation.

As described above, in the absence of cellular stress, most of the serines and threonines of the p53 TAD are unphosphorylated. In particular, the absence of phosphorylation of Thr18 allows tight binding of Mdm2 to suppress p53 activity by enhancing nuclear export and proteosomal degradation. Once a cell experiences a stress, the concentration of p53 rapidly rises to stimulate the appropriate response: e.g. cell cycle arrest or apoptosis. One measure of the speed of response is that phosphorylation of Ser15, a marker of p53 activation, is detectable within 30 min after exposure to IR (74). This rapid change necessitates a switch-like inhibition of the interaction with Mdm2. As in vitro experiments have shown, the binding affinity of the TAD1-Mdm2 complex can be reduced 5- to 25-fold solely by phosphorylation of Thr18 (60–62). Mutational studies have demonstrated that the effect results from electrostatic repulsion of negatively charged pThr18 by a proximal patch of acidic and aromatic residues on the surface of the Mdm2 domain (Figure 5A) (75,76).

In contrast, the interactions of p53 with its positive cofactors generally start out weak and increase in affinity with increasing phosphorylation. This allows for a nuanced response in which the interactions of p53 with different subgroups of cofactors change over time. This phenomenon has been best demonstrated for the interaction with CBP/p300 (59–64). As shown in Figure 3B, the strength of the effect depends on the location of the phosphorylation within the TAD sequence and varies for the different domains of CBP/p300. Single phosphorylation of Ser15, Thr18, Ser20, Ser33, Ser37 or Ser46

Fig. 4. Hydrophobic interactions in p53 complexes. In each structure, p53 is shown as a blue ribbon and the binding partner is shown as gray surfaces. Hydrophobic residues from p53 are highlighted as magenta spheres. (A) Mdm2 (PDB: 1YCQ); (B) p300 Taz2 (PDB: 2K8F); (C) CBP NCBD (PDB: 2L14); (D) Tfb1 (PDB: 2GSO); (E) RPA70 (PDB: 2B3G) PDB, Protein Data Bank.
generally increases the binding affinities to the Taz1, Taz2 and KIX domains by 2- to 7-fold, whereas the effects on the NCBD are smaller (63,67). Although the latter interaction was confirmed with alanine mutants, the binding affinity of TAD1-pSer15 was unaffected by mutation of Arg1737 (63). As Ser15 is in a more flexible region of the peptide than Thr18, the phosphorylated residue could shift to bind a different amino acid of Taz2 or, alternately, the entire TAD1 could bind in a different orientation. Three observations support these possibilities (63,65): (i) the affinity of diphosphorylated TAD1 is intermediate between the affinities of the corresponding monophosphorylated forms; (ii) changes in amide $^1$H and $^1$N chemical shifts measured by NMR indicate conformational changes in Taz2 when Thr18 is phosphorylated; (iii) differences in the temperature dependence of the heat capacity suggest that stabilization of the mono- and diphosphorylated forms arises primarily from hydrophobic and electrostatic interactions, respectively. Determination of the NMR solution structures of the complexes with phosphorylated p53 is hampered by the difficulty and expense of producing isotopically labeled phosphopeptides.

In contrast to the TAD1-Taz2 complex, analysis of the structure of unmodified TAD bound to the NCBD (Figure 4C) failed to suggest proximal positively charged residues that could form stabilizing salt bridges, an observation consistent with the relatively weak effect of phosphorylation on binding (Figure 3B). Additionally, negatively charged residues on the surface of the NCBD that could explain the reduced affinity upon Thr18 phosphorylation were not observed. Therefore, the moderate changes in affinity that result from p53 phosphorylation likely reflect a structural rearrangement of the TAD-NCBD complex.

Modification of TAD2 enhances the binding of p53 to p62, with monophosphorylation of Ser46 or Thr55 each increasing the affinity of TAD2 for p62 6-fold and the diphosphorylated form binding 32-fold tighter (78). As in the TAD1-Taz2 complex, the structure of the unmodified complex suggests positively charged residues that could form stabilizing interactions with the phosphorylated residues. In the Tfb1 complex, pSer46 of TAD2 is adjacent to Lys11 of Tfb1 and pThr55 is proximal to Arg61 (Figure 5C). Interaction of the former was confirmed by mutagenesis of the corresponding residue in p62 (78). In contrast, the residue in p62 that corresponds to Arg61 in Tfb1 is Glu66, which lacks a formal charge. Instead, pThr55 may interact with Lys54 of p62, which corresponds to Gln49 in Tfb1 and is adjacent to Arg61 (Figure 5C). The physiological implication of the increased binding of pThr55 is unclear, as this modification is associated with repression of p53.

Less is known about the effects of p53 phosphorylation on interactions with proteins that bind p53 as a single-stranded DNA mimic. For the interaction of the TAD with PC4, hepta-phosphorylated p53 increased the affinity to the net positively charged PC4, consistent with the ability of this cofactor to activate p53 (73). In contrast, UV exposure abrogates the interaction of p53 with RPA, suggesting that phosphorylation may reduce the affinity of the complex (79). Based on the negative regulatory role of BRCA2 binding to p53, phosphorylation may also repress this interaction. The structure of the complex of p53 TAD with RPA70N (Figure 4E) does not suggest any formally charged residues that could interact with phosphorylated residues in TAD2.

The effects (loss or gain) on p53 transcriptional activity observed in the knock-in mice are consistent with the role of these sites on binding of cofactors. Phosphorylation generally increases the affinity of p53 for its positive regulators; serine to alanine mutations that block phosphorylation decreased p53 transcriptional activity, probably due in part to the decreased affinity of the mutant p53 for critical cofactors. Additionally, the increased defects in p53-dependent stress responses in p53$^S18A,S23A/S18A,S23A mice as compared with p53$^S18A/S18A and p53$^S23A/S23A knock-in mice are consistent with the additive effects of p53 TAD1 phosphorylation on binding.
**Concluding remarks**

One of the interesting questions raised by these studies is why p53 contains two similar acidic transactivation subdomains. Although both contain \( \Phi-X-X-\Phi-\Phi \) motifs, the surrounding residues differ. TAD1 contains seven modifiable serine or threonine residues, whereas TAD2 contains only two. TAD1 contains seven acidic residues (18% of the total domain) with an estimated pI of 3.71 in the unmodified state; TAD2 contains nine acidic residues (35% of the total domain) with an estimated pI of 3.10. Thus, in the unstrained state, TAD2 presents a greater density of charged residues than TAD1; following p53-activating stress, however, the two subdomains become approximately equally charged. The nine phosphorylation sites within the full TAD are modified by enzymes involved in different signaling pathways. Moreover, the same site can be modified by multiple kinases. For example, Ser15 can be phosphorylated by kinases that respond to DNA damage, nutrient deprivation, hormone stimulation and hypoxia. Specific stresses result in distinct patterns of phosphorylation over time (80). Differences in the charge of the subdomain and pattern of modification give rise to specificity in the protein–protein interactions in which the subdomains participate. Although many of the domains of CBP/p300 interact with both TAD1 and TAD2, Mdm2 only binds to TAD1, whereas RPA70 and p62 only bind to TAD2 (69,78). Thus, the two subdomains provide extra flexibility for p53 to respond to different stress signals and to mediate the multiple responses required by the specific stress in a cell- or tissue-type-dependent manner. This is exemplified by \( \Delta Np53 \) (an isoform of p53 in which translation is initiated at Met40 such that it contains only TAD2), which has specific functions in stem cells, embryonic development and following endoplasmic reticulum stress (81–83).

The different functional effects of Ser15 and Ser46 phosphorylation also demonstrate the differences between the TAD subdomains. When aligning TAD1 and TAD2 by their \( \Phi-X-X-\Phi-\Phi \) motifs, Ser15 correlates to Ser46; phosphorylation of these two residues might be expected to exhibit similar regulation and function. With the sole exception of the adenosine monophosphate-activated protein kinase (AMPK), which can directly phosphorylate both, the two sites are specifically modified by several different kinases, none of which has been shown to directly phosphorylate the other site. In addition, distinct phosphatases remove the modifications. One recent study demonstrated that the amount of p53 phosphorylated at Ser15 bound to DNA was similar following treatment of U2OS osteosarcoma cells with actinomycin D to induce cell cycle arrest or etoposide to induce apoptosis; in contrast, DNA-bound p53 phosphorylated on Ser46 increased 5-fold after etoposide treatment as compared with actinomycin D treatment (84). Thus, the regulation and functional outcome of phosphorylation of these two sites are quite different, indicating that they play unique roles.

Another aspect of the presence of two TAD subdomains of p53 is that it allows the formation of ternary complexes, such as those observed between p53, CBP/p300 and Mdm2 (61,63). Although these form on a single molecule of p53 using isolated domains in vitro, the sizes of the intact proteins would likely sterically preclude the same from occurring in vivo. However, as p53 forms a tetramer in the nucleus, it is possible that Mdm2 could bind one monomer of p53 while CBP/p300 binds either subdomain on a separate monomer. Such a ternary complex may represent an intermediate state early after stress. In experiments with nutlin-3a, an inhibitor of the TAD1-Mdm2 interaction, CBP/p300-dependent acetylation of the REG domain was observed in the absence of TAD1 phosphorylation (85,86). These results suggest that upon removal of the inhibitory effect of Mdm2, p53 is able to accumulate to a level comparable with that following stress, which allows critical protein–protein interactions to form in the absence of phosphorylation.

The stability and activity of p53 are very tightly controlled by phosphorylation. Within the TAD, phosphorylation can have either a positive or negative effect on p53 stability, activity or both. The same trends are observed in the REG domain, in which phosphorylation, acetylation, ubiquitination and ubiquitination are all found. As the same lysine residues can be methylated, acetylated and ubiquitinated, the complexity of p53 regulation quickly rises. In addition, N-terminal modifications can affect C-terminal ones. For example, phosphorylation of the TAD promotes acetylation by CBP/p300 and negatively regulates ubiquitination and sumoylation in the REG domain (87). As illustrated here, p53 posttranslational modifications can modulate protein–protein interactions; additionally, they can affect p53 tetramerization, which has direct implications for both stability and activity. Intriguingly, p53 polymorphisms have recently been shown to also affect modification, with p53 Arg72 showing enhanced phosphorylation of Ser6 and Ser20 as compared with p53 Pro72 (88).

Clearly, the regulation of p53 protein level is critical. It must be stabilized in response to stress to protect the cell, as evidenced by the hightumor rate among p53−/− mice. However, following removal of the stress and clearance of the resulting damage, p53 levels must return to steady-state levels: Mdm2−/− mice are embryonically lethal and overactive p53 leads to an accelerated aging phenotype. p53 messenger RNA levels are generally static; thus, the primary regulation of p53 stability and activity is through the modulation of protein interactions by posttranslational modification.

Although much has been done to identify the sites and effects of p53 posttranslational modifications, there are gaps in our understanding. Most of the research on the modulation of protein–protein interactions by phosphorylation has necessarily been done in vitro; in the future, experiments should be performed to analyze these complexes in cells as well. In addition, more effort should be devoted to unraveling the interplay between different sites of modification, such as how modification on one site affects the modification of a second site. Signaling cascades among N-terminal phosphorylation sites have been studied following different stresses (80), but the interplay of modifications in the REG domain of p53 and how they are affected by N-terminal phosphorylation have received less focus. Finally, it will be critical to explore the effect of posttranslational modification on new functions of p53, including metabolism. Combined these studies will lead to a new era in the understanding of the complex layers of p53 regulation.

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p53 TAD phosphorylation


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