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News and Commentary

Regulation of p53 responses by post-translational modifications

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The p53 gene is the most commonly mutated tumor suppressor gene in human cancers. It has been well established that p53 plays multiple tumor suppression roles in cells upon introduction of stresses: cell cycle G_1 and G_2 arrest or apoptosis, in part depending on the cell types.[1] Structural and functional analyses of p53 have shown that p53 is a transcription factor with a sequence-specific DNA-binding domain in the central region and a transcriptional activation domain at the N-terminal.[1] Three additional domains, including a nuclear localization signal, a tetramerization domain and an extreme C-terminal regulatory domain, are present in the C-terminal of p53.

The transcriptional activities of p53 are essential for its tumor suppression functions. p21, a downstream target of p53 and a universal inhibitor of cyclin-dependent kinases, plays a major role in mediating p53-dependent cell cycle G_1 arrest.[2–4] While earlier studies reached conflicting conclusions on the requirement of p53 transcriptional activities in p53-dependent apoptosis,[1] recent analysis of knockin mice and cells, in which two missense mutations encoding Gln and Ser in place of Leu25 and Trp26 were introduced into the endogenous p53 gene, indicated that p53 transcriptional activities are essential for p53-dependent apoptosis.[5,6] Therefore, identification of genes whose expression is either activated or repressed by p53 will be essential in order to understand the mechanism of p53-dependent tumor suppression.

In response to DNA damage and other cellular stresses, the protein levels of p53 are greatly upregulated and its activities induced. It had been postulated that p53 was present in an inactive form in normal cells in the absence of stress.[1] In this context, the extreme C-terminal of p53 was proposed a role in negatively regulating the sequence-specific DNA binding of p53.[7–9] However, evidence provided by two recent studies argued against such a model and indicated that p53 could bind to its sequence-specific DNA-binding site in cells in the absence of stress.[10,11] Therefore, it remains to be elucidated how p53 activities are induced after introduction of stress. The induction of p53 protein levels post stresses is regulated post-translationally and mainly because of increased protein stability.[1] MDM2, a transcriptional target of p53, plays a major role in the regulation of p53 stability.[12,13] MDM2 interacts with the N-terminal of p53 and this interaction could inhibit p53 transcriptional activity.[14] In addition, MDM2 acts as the E3 ligase for p53 and thus promotes p53 ubiquitination and degradation.[15–19] Therefore, MDM2-p53 interaction represents a negative autoregulatory mechanism to regulate p53 stability and activity. Accumulating evidence has indicated that post-translational modifications of p53 and MDM2, including phosphorylation, acetylation and sumoylation, could modulate their interaction and thus p53 stability and activity.

Roles of p53 phosphorylation

Human p53 is phosphorylated at multiple sites at the N- and C-terminal *in vitro* by a number of kinases, and most of these phosphorylation sites are conserved between human and mouse p53 (Table 1). However, several phosphorylation sites within the proline-rich domain are not conserved between human and mouse p53. For example, Ser46 of human p53 can be phosphorylated by a number of kinases after DNA damage (Figure 1a;[20–23]). There is no apparent mouse homolog of human Ser46.

Several p53 phosphorylation events have been extensively studied. ATM family kinases are required for the rapid phosphorylation of human p53 at Ser15 following DNA damage (Figure 1a;[24–26]). One study reported that phosphorylation of p53 at Ser15 disrupted its interactions with MDM2, leading to p53 stabilization.[27] An additional study suggested that Ser15 phosphorylation might stabilize p53 by preventing p53 nuclear export.[28] However, others have presented evidence arguing that Ser15 phosphorylation did not disrupt MDM2–p53 interaction, but was required for p53 acetylation and activation.[29–31] To address the physiological significance of Ser15 phosphorylation in the p53 responses to DNA damage, a missense mutation was introduced into the endogenous p53 gene in mice that changed the nucleotides encoding Ser18 (corresponding to Ser15 of human p53) to those encoding alanine. Analysis of the homozygous $p53^{Ser18Ala}$ ES cells and differentiated ES cells indicated that phosphorylation of mouse p53 at Ser18 is required for the maximum p53 responses to DNA damage.[32] However, in contrast to the previous studies suggesting that phosphorylation of human p53 at Ser15 was required for p53 acetylation after DNA damage, analysis of p53 acetylation in $p53^{Ser18Ala}$ differentiated ES cells indicated that the phosphorylation of p53 at Ser18 was neither necessary nor sufficient to activate p53 acetylation at C-terminal.[5,32] One possible explanation for this discrepancy is that the requirement of Ser18 phosphorylation in p53 acetylation is cell-type specific. Analysis of DNA damage-induced p53 acetylation in other cell types derived

Table 1 Summary of various phosphorylation and acetylation events of human (hp53) and mouse p53 (mp53), as well as their potential roles in regulating p53 stability and activity

Phosphorylation sites

Human p53	Corresponding mouse p53	Potential functions of the modification	References
Ser15	Ser18	Disruption of p53–MdM2 interaction Recruitment of CBP/p 300 p53 acetylation and nuclear retention	51,28–30
Ser20	Ser23	Disruption of p53–MdM2 interaction	33–35,37,39,52
Ser33	N/A	Activation of p53 activities after	20,44,53,54
Ser46	N/A	UV radiation	
N/A	Ser34	N/A	55
Thr81	N/A	Stabilization of p53 after UV radiation	53
N/A	Thr76/86	N/A	56
Ser315	Ser312	Regulation of p53 activities during cellular proliferation and DNA damage	57–59
Ser376/378	Ser373/375	Activation of p53 activity through promoting p53/14-3-3 interaction after IR	60
Ser392	Ser389	Regulation of p53 responses to UV radiation?	61–67
Acetylation sites			
Lys320	Lys317	N/A	42,44,45
Lys370/372/373/381/382/386	Lys367/369/373/378/379/383	Recruitment of coactivators CBP/p300 after DNA damage. Stabilization of p53 after DNA damage	30,42,43,46,49,50

from p53^{Ser18Ala} mice could test this possibility. Alternatively, it is possible that the mechanism regulating p53 acetylation is different between humans and the mouse.

Ser20 of human p53 and corresponding Ser23 of mouse p53 are phosphorylated *in vivo* following DNA damage.[13,33] This phosphorylation event is mediated by Chk2 kinase, which is activated by ATM family kinases after DNA damage (Figure 1a;[33–36]). Several studies have suggested that phosphorylation of human p53 at Ser20, which is located within the α helix involved in MDM2 interaction, is important for p53 stabilization after DNA damage by disrupting MDM2–p53 interaction.[33–35] However, several other studies indicated that phosphorylation of human p53 at Ser20 did not affect MDM2-p53 interaction.[37,38] To address the physiological significance of phosphorylation of mouse p53 at Ser23 in p53 responses to DNA damage, knockin ES cells, in which the missense Ser23 (corresponding to Ser20 of human p53) to Ala mutation was introduced into the endogenous p53 gene, were generated. Studies of the p53^{Ser23Ala} ES cells, embryonic fibroblasts and thymocytes indicated that phosphorylation of mouse p53 at Ser23 was not required for p53 stabilization after DNA damage.[39] It is possible that Ser23 phosphorylation is required for more subtle responses to DNA damage and this will await future analysis of p53^{Ser23Ala} germline knockin mice. This finding suggests that, in the absence of Ser23 phosphorylation, other phosphorylation events of p53 could stabilize p53 to normal levels. Consistent with this notion, phosphorylation of Thr18, which is also located within the region of p53 interacting with MDM2,[40] could interrupt p53–MDM2 interaction leading to p53 stabilization. Several possibilities can be considered to explain the discrepancy between this conclusion and the earlier ones, which indicated an important role of Ser20 phosphorylation in the disruption of p53–MDM2 interaction. First, it is possible that phosphorylation of human p53 at Ser20 and mouse p53 at Ser23 have different roles in regulating p53 stability and activity. However, several lines of evidence argued against this possibility. The human p53 knockin mice, in which the core region (amino-acid sequence 33–332) of the endogenous mouse p53 was replaced with the human counterpart, have normal p53 responses to DNA damage and p53-dependent tumor suppression.[41] This indicates that the DNA-damaged-induced signaling pathways leading to the activation of p53 responses are highly conserved between human and the mouse. In addition, the amino-acid sequence between 13 and 27 (human numbering) is identical between human and mouse p53. Secondly, most previous assays involved the overexpression of mutant p53 in tumor cell lines. It is possible that tumor cells are defective in certain signaling pathways leading to the phosphorylation of p53 at other sites, contributing to the more apparent requirement of Ser20 phosphorylation in p53 stabilization.

A number of other phosphorylation events of mouse and human p53 have been identified after DNA damage or during cellular proliferation. The kinases involved in these phosphorylation events and the potential roles of these phosphorylation events in regulating p53 stability and activity are summarized in Table 1.

Roles of p53 acetylation

CREB-binding protein (CBP) and p300 can coactivate numerous transcriptional factors, including p53.[30,42,43] Both PCAF and p300 possess intrinsic histone acetyltransferase activity and can acetylate human p53 at Lys320 and Lys372/

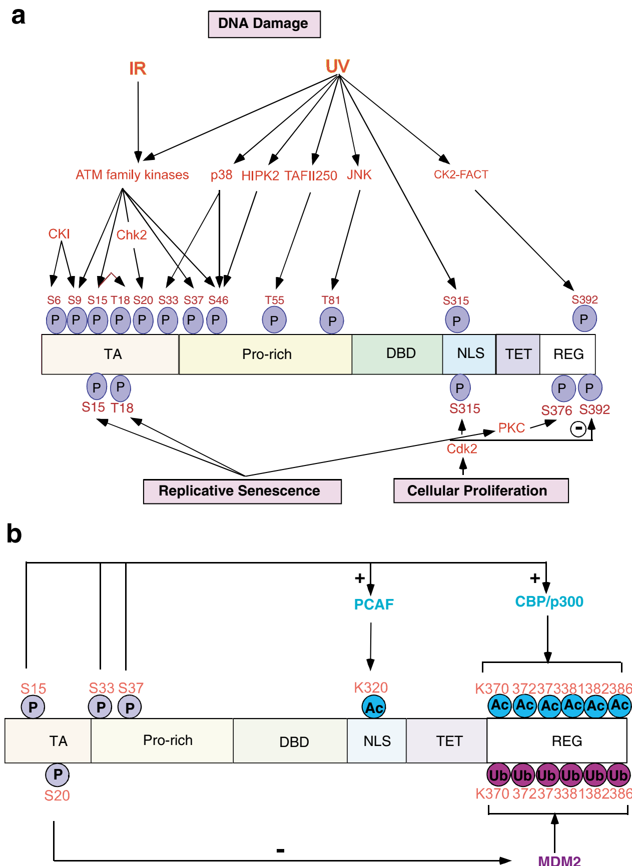

Figure 1 (**a**) Phosphorylation of human p53 upon genotoxic stresses or during cellular proliferation and senescence. Phosphorylation at Ser15 is required to activate phosphorylation at Thr18 after DNA damage. IR, ionizing radiation; UV, ultraviolet radiation; P, phosphorylation; S, Serine; T, Threonine; ATM, Ataxia-telangiectasia Mutated; p38, p38 MAP kinase; HIPK2, homeodomain-interacting protein kinase 2; JNK, JUN-terminal kinase; CK1 and CKII, casein kinase 1 and 2; FACT, chromatin transcriptional elongation factor (hSpt16 and SSRP1 heterodimer); CDK2, cyclin-dependent kinase 2; PKC, protein kinase C. (**b**) Functional interaction between p53 phosphorylation, acetylation and ubiquitination. Phosphorylation of p53 at Ser15, Ser33 and Ser37 might activate p53 acetylation by increasing the interaction between p53 and coactivators PCAF and CBP/p300. Phosphorylation of p53 at Ser20 might disrupt the interaction between p53 and MDM2, thus inhibiting MDM2-dependent ubiquitination of p53. The C-terminal lysine residues acetylated after DNA damage are also the residues ubiquitinated by MDM2. P, phosphorylation; AC, acetylation; Ub, ubiquitination.

373/381/382, respectively (Figure 1b;[42,44,45]). In addition, these lysine residues of p53 are also acetylated in response to DNA damage *in vivo*.[44,45] Several mechanisms have been proposed to account for the regulation of p53 acetylation at C-terminal. DNA damage-induced phosphorylation of p53 might regulate the interaction between PCAF/p300 and p53. In support of this notion, two studies suggested that phosphorylation of p53 at Ser15 or Ser33/37 promotes the interaction between p53 and CBP/p300 (Figure 1b;[31,44]). Secondly, MDM2, which destabilizes p53 by promoting p53 ubiquitination, might also suppress p300-mediated p53 acetylation by forming a complex with p300 and p53 (Figure 1b;[46]). Thirdly, tumor suppressor PML could induce p53 acetylation through the formation of a trimeric p53–PML–CBP complex.[47]

While an earlier study suggested that the acetylation of p53 at C-terminal Lys residues can activate p53 sequence-specific DNA-binding activity *in vitro*,[42] human p53 with mutations at multiple acetylation sites at the C-terminal (Lys372,373,381,382 to Ala) appears to be functional in both sequence-specific DNA-binding and transactivation.[48,49] In addition, recent analysis of the DNA-binding activities of a p53 mutant, which harbored multiple Lys to Arg mutations at the acetylation sites, indicated that p53 acetylation by p300 was not required for sequence-specific DNA binding activities of p53.[10] However, this study also indicated that the C-terminal of p53 and recruitment of p300 to the p53-dependent promoters were required for p53 transcriptional activities. The involvement of p53 acetylation in activating its transcriptional activities was suggested by the findings that p53 acetylation was required for the recruitment of CBP/p300 to the p53-dependent promoters.[50]

Another potential role of p53 acetylation is to regulate p53 stability. While MDM2 binds to the N-terminal of p53, the extreme C-terminal region of p53 (362–392) is also required for the efficient MDM2-mediated degradation.[15] Several recent studies have managed to identify the elements within the extreme C-terminal of p53 that might be involved in regulating MDM2-mediated p53 degradation. By mutating the six Lys residues (370, 372, 373, 381, 382 and 386) to Arg at the C-terminal, one study showed that these mutations (K6R) essentially abolished the MDM2-mediated ubiquitination of p53, suggesting that these lysine residues are the ubiquitination sites.[49] Consistent with this finding, another group mutated four lysine residues 370, 373, 381 and 382) into Ala (K4A) and the K4A mutation also impaired the MDM2-mediated ubiquitination.[48] MDM2 bound to K6R and K4A p53 mutants equally well when compared to the wild-type p53, indicating that the defective p53 ubiquitination was not because of the impaired MDM2–p53 interaction. Since the C-terminal lysine residues ubiquitinated by MDM2 are the same residues for acetylation after DNA damage, acetylation of these lysine residues after DNA damage could prevent ubiquitination at the same sites, leading to p53 stabilization.

Future directions

Recent studies have shown that phospho- or acetylation-specific antibodies, which recognize p53 modified at a particular site, are indispensable tools to study the regulation and interaction among various post-translational modification events. Development of a complete set of these antibodies to modified mouse and human p53 in the future will be critical to identify the unique patterns of post-translational modifications induced by a particular stress stimulus and the interactions between the various post-translational modifications. Overexpression of p53 mutants in tumor cell lines is a risky experimental approach and the conclusions from such studies will require verification for their physiological relevance. Studies employing mouse knockin technology could address the physiological roles of p53 post-translational modifications, but will represent a major effort. Some phosphorylation sites within the proline-rich domain of mouse and human p53 are not conserved. The physiological

importance of these unconserved phosphorylation events of human p53 could now be tested using the humanized p53 knockin mouse model, in which the core region of human p53 (33–332) has replaced the mouse counterpart.[41] However, this requires that the stress-induced signaling pathways leading to these phosphorylation events be conserved between the mouse and human cells. Finally, identification of the phosphorylation events and subsequently the signaling pathways required for p53 activation in response to DNA damage will have important clinical applications, including a screen for defects in these signaling pathways in tumor cells expressing a wild-type but dysfunctional p53.

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