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Review

Post-translational modifications of p53 tumor suppressor: determinants of its functional targets

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Summary. Tumor suppressor p53 functions as a "guardian of the genome" to prevent cells from transformation. p53 is constitutively ubiquitinated and degradated in unstressed conditions, thereby suppressing the expression. However, cellular stimuli enable p53 to escape from the negative regulation, and then stably expressed p53 transactivates its target genes to induce cell cycle arrest, DNA repair, or apoptosis. Promoter preference of target genes is determined by modification status of p53. Because p53 has two critical roles in the decision of cell fate, stopping cell cycle to repair damaged DNA or induction of apoptotic cell death in response to DNA damage, elucidation of switching mechanisms on p53 functions is of particular importance. Here we review recent evidence how several post-translational modifications of p53 including methylation, phosphorylation, acetylation, and ubiquitination, affect the functions of p53 in response to cellular stress.

Key words: p53, Apoptosis, Cell cycle arrest, DNA damage

Introduction

The maintenance of genome stability and proper cell cycle control are essential for cellular function and survival in all organisms. Dysfunctions of these regulations are involved in inherited genetic disorders in aging and tumorigenesis. In particular, tumorigenesis arises from functional attenuation of tumor suppressor genes that regulate genome stability and cell cycle progression. Tumor suppressor p53 functions as a "guardian of the genome" and regulates cell cycle checkpoint, DNA repair, and induction of apoptosis in response to DNA damage. Since mutations of p53 are found in more than half of human cancers, the understanding of p53 functions would contribute to the construction of new cancer treatments. p53 is divided into three functional domains, N-terminal transactivation domain, central core sequence-specific DNA-binding domain, and C-terminal regulatory domain (Bode and Dong, 2004; Kruse and Gu, 2009). The N-terminal transactivation domain is required for regulating stability and transcriptional activity of p53. To be more specific, phosphorylations of the N-terminal transactivation domain modulate the affinity for its negative regulator, MDM2, and the selective transactivation of p53 target genes. The central core sequence specific DNA-binding domain is needed for the recognition of the promoters in p53 target genes. Almost all reported mutations of p53 are located in this domain, resulting in loss of DNA binding capability. The C-terminal regulatory domain possesses nuclear localization signal (NLS), nuclear export signal (NES) as well as tetramerization domain, and is regulated by many post-translational modifications, including ubiquitination, acetylation, and methylation. Under unstressed conditions, p53 expression level is suppressed due to being constitutively ubiquitinated by RING-finger ubiquitin E3 ligase, MDM2, and degradated via proteasome. In response to genotoxic stress, p53 is stabilized to up-regulate the target genes involved in cell cycle arrest and DNA repair. Furthermore, when DNA damage is irreversible, p53 induces expression of pro-apoptotic genes (Yoshida and Miki, 2010). Selective transactivation of target genes is determined by post-translational modifications of p53. This review is

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focused on the p53 post-translational modifications that determine the selective transactivation of its target genes in response to DNA damage.

Methylation

Methyltransferase catalyses a transfer of methyl group to lysine (Lys) or arginine (Arg) residues of its substrate. Methylations of histones are involved in up- or down-regulation of transcription, as perceived from the "histone code" hypothesis. Methylation of non-histone proteins, such as p53, is a vital post-translational modification for regulating protein function. In the case of p53, methylations of the Lys residues in the C-terminal regulatory domain, as in histone methylations, function to regulate p53 function positively or negatively. Set7/9 histone-lysine N-methyltransferase methylation of p53 at Lys372 up-regulates expression of p53 target genes (Chuikov et al., 2004). In contrast, methylation of Lys382 by Set8 histone-lysine N-methyltransferase suppresses the transcriptional activity of p53 (Shi et al., 2007). After DNA damage, Set8 expression is attenuated, allowing acetylation of p53 by p300. Lys382 acetylation enables p53 to bind to the promoter regions of target genes. SET and MYND domain containing 2 (Smyd2)-mediated Lys370 methylation negatively regulates p53-induced transcription by inhibiting its recruitment to DNA. Following DNA damage, Set9 methylation of p53 at Lys372 inhibits Smyd2-mediated Lys370 methylation, thereby promotes p53 binding to the promoters of *p21* and *mdm2* genes (Huang et al., 2006). Furthermore, demethylation of p53 at Lys370 by the lysine-specific demethylase KDM1 (LSD1) attenuates the interaction with its co-activator p53-binding protein 1 (p53BP1), resulting in inhibition of pro-apoptotic function of p53 (Huang et al., 2007). Therefore, Lys370 and Lys382 methylations have an inhibitory effect on transcription activity of p53 (Fig. 1). These Lys residues are also acetylated by p300 to increase p53 activity (Gu and Roeder, 1997). Moreover, these residues serve as ubiquitination sites by MDM2 (Lohrum et al., 2001). Hence, combination of these modifications could determine multiple p53 functions. On the other hand, the protein arginine methyltransferase 5 (PRMT5) methylates three Arg residues (Arg333, Arg335 and Arg337) of p53, which were located in the oligomerization domain. The methylations affect not only the cellular localization and oligomerization but also promoter binding (Jansson et al., 2008). Taken together, Arg and Lys methylations of p53 are involved in the regulation of its transcriptional activity. Interestingly, Lys370 and Lys382 residues are common modification sites for methylation and acetylation. Through these competitions, p53 acquires rapid activation upon stimuli. In addition, these Lys residues can also be recognized by MDM2 to target p53 for degradation (Lohrum et al., 2001). At present, it is not clear how ubiquitination of these Lys sites integrates into the p53 activation/suppression regulatory machinery. The clue to the answer of this question could be provided by the comparison of p53 modifications under various types of DNA damage. A recent study demonstrated that p53 acetylation levels, under transient DNA damage, are low compared to those under sustained damage (Loewer et al., 2010). Since these Lys residues can be ubiquitinated, low acetylated p53 is allowed to be ubiquitinated by MDM2. Given that

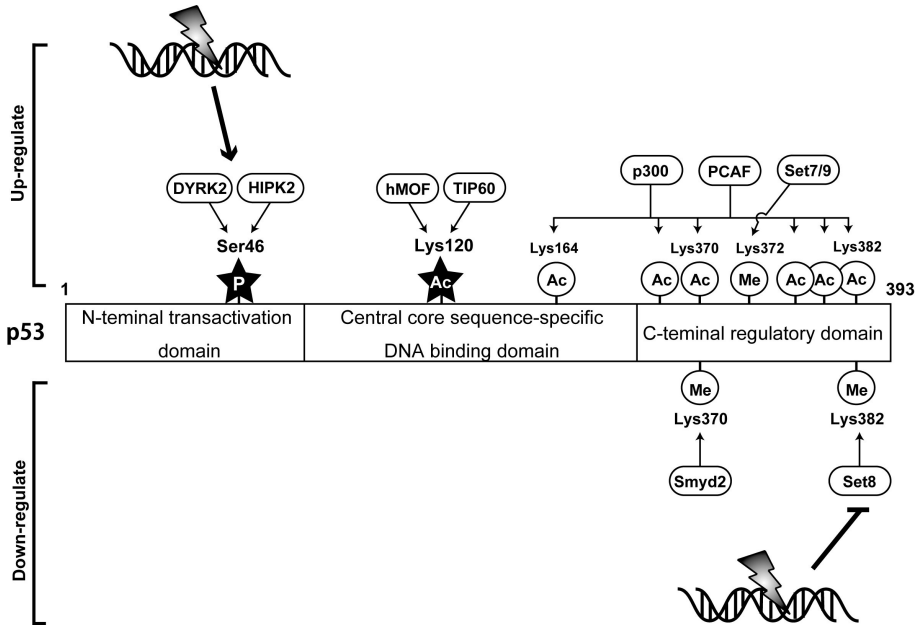

Fig. 1. Overview of critical modification sites for p53 function. p53 has three functional domains; N-terminal transactivation domain, central core sequence specific domain, and C-terminal regulatory domain. Upper diagram indicates modification sites for up-regulating p53 function. Star marked residues are requisite for apoptosis induction. Lower diagram shows down-regulating modifications for p53 function. P, phosphorylation; Ac, acetylation; Me, methylation.

attenuated p53 expression contributes to re-start cell cycle, unmodified Lys residues could be targeted for ubiquitination to resume cell cycle. In contrast, upon sustained or irreversible DNA damage, p53 is acetylated to arrest cell cycle and to induce apoptosis. Thus, Lys acetylation is required for, not only activating p53 function, but also inhibiting MDM2-mediated ubiquitination. Taken together, the balance of p53 modifications between acetylations and ubiquitinations could determine cell fate (Berger, 2010). However, methylation status of Lys residues under same conditions remains unclear. Monitoring methylation levels provides a clue to understand the complicated switch of these modifications.

Phosphorylation

Phosphorylations of p53 at serine (Ser) /threonine (Thr) /tyrosine (Tyr) residues are indispensable for the signal transduction in response to DNA damage. Phosphorylations of the N-terminal transactivation domain are a particular prerequisite for p53 stabilization and selective transactivation of p53 target genes. As described above, p53 is constitutively ubiquitinated and degradated under normal conditions. Once cells are exposed to genotoxic agents, p53 is phosphorylated at the N-terminal transactivation domain by several kinases, resulting in an increment of expression. Ser15 and Ser20 phosphorylations by ataxia telangiectasia mutated (ATM) (Shieh et al., 1997) and checkpoint kinase 1/2 (Chk1/2), respectively, enable p53 to escape from the MDM2-mediated ubiquitination. Stablilized p53 transactivates its target genes to promote cell cycle arrest (e.g., p21, 14-3-3Ũ) followed by repair DNA of lesion (e.g., p53R2) (el-Deiry et al., 1993; Hermeking et al., 1997; Tanaka et al., 2000). Under severe DNA damage, Ser46 is additionally phosphorylated by Ser46 kinase(s) to induce p53-mediated apoptosis-related genes, such as p53-regulated apoptosis-inducing protein 1 (p53AIP1) (Oda et al., 2000; Yoshida et al., 2006). After gamma ray irradiation, p53AIP1 localizes at the mitochondria and induces apoptosis. Thus, identification of the Ser46 kinase(s) is important for switching mechanism of p53 function. Moreover, it could be a molecular target for cancer therapy since phosphorylated p53 is an efficient apoptosis inducer in tumor cells. Two groups reported that Homeodomain-interacting protein kinase 2 (HIPK2) phosphorylates p53 at Ser46 in response to UV–irradiation (D'Orazi et al., 2002; Hofmann et al., 2002). In contrast, our recent study demonstrated that dual specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2) phosphorylates p53 at Ser46 in response to both adriamycin- and UV-induced DNA damage (Taira et al., 2007; Yoshida, 2008) (Fig. 1). Furthermore, our results showed that HIPK2 contributes to UV- but not adriamycin-mediated p53 phosphorylation at Ser46. At present, it is unclear whether a role of DYRK2 in response to UV-irradiation is dependent on, competing with, or redundant with HIPK2. Nevertheless, these reports demonstrated the importance of Ser46 modification using a p53 mutant. A knock-in mice model showed that a p53-dependent apoptosis is suppressed in the cells from mice expressing the human p53 genes with the S46A mutation, compared to the wild type p53 knock-in mice (Feng et al., 2006). On the other hand, a p53-46F mutant, in which Ser46 is replaced with Phe, strongly induces p53 target genes and apoptosis (Nakamura et al., 2006). However, it has not been clarified whether Ser46 substitution with Phe mimics to the phosphorylated form of p53. These findings suggested that the transduction of p53-46F mutant would be useful for p53-defective tumor treatment. Moreover, introduction of the p53 mutant p53-46D, which mimics Ser46 phosphorylation, can overcome resistance to p53 induced apoptosis in human HSC-3 oral cancer cells (Ichwan et al., 2006). Taken together, phosphorylation of p53 at Ser46 is a critical modification for p53 to direct apoptotic cell death.

Acetylation

The covalent binding of acetyl group to Lys residue was first found in histone protein. Similar to methylation, acetylation of histone proteins is a requisite for the basal activation or suppression of transcription. p53 is the first non-histone acetylated protein identified and is functionally regulated by acetylation and deacetylation (Gu and Roeder, 1997). Lys residues in the p53 C-terminal regulatory domain up-regulate DNA binding capability and transcriptional activity of p53 through efficient recruitment of co-factors in response to several stresses. Upon UV and ionizing irradiation, Lys320 is acetylated by PCAF to specifically bind to the DNA (Liu et al., 1999). Gu and Roeder demonstrated that p300 functions as a co-activator and acetylates p53 C-terminal Lys residues, triggering p53 recruitment to the DNA (Gu and Roeder, 1997). One model for how acetylation enhances p53 DNA binding is that acetylations induce p53 conformational changes through neutralizing the positively charged C-terminal domain (Gu and Roeder, 1997; Sakaguchi et al., 1998). Furthermore, these p300 and PCAF-mediated p53 acetylations also play an important role in recruiting co-factors to p53 target genes. Mutation of these acetylation sites inhibits interaction with co-factors, TRRAP and TAFII30, as well as histone acetyl transferases (Barlev et al., 2001). In addition to these C-terminal acetylations, two Lys residues in the p53 central DNA-binding domain are reported to be acetylated. These Lys residues are highly conserved and often mutated in human cancer. One of these acetylation sites, Lys120, is acetylated by hMOF and TIP60, thereby up-regulating expressions of pro-apoptotic target genes such as puma and bax, but not p21 and MDM2 (Charvet et al., 2011; Sykes et al., 2006; Tang et al., 2006). Another acetylation site, Lys164, is also catalyzed by p300. Mutations of these two and the C-terminal six Lys residues (p53-8KR) abrogate p53

transcriptional activity and cell cycle progression, although a p53-8KR mutant retains the DNA-binding ability. These data suggest that these acetylations affect p53 promoter selectivity and the tumor suppressive function of p53 (Tang et al., 2008). Collectively, acetylations of p53 positively regulate its function (Fig. 1). As expected, deacetylations by HDAC1 and SIRT1 attenuate p53-dependent transcription and its tumor suppressive functions (Luo et al., 2001, 2001). These reports suggest that deacetylation is employed as an alternative MDM2 independent mechanism for inhibition of p53 function. The C-terminal Lys residues are competition sites for MDM2-mediated ubiquitination (Li et al., 2002). Under unstressed conditions, MDM2 negatively regulates p53 function. On the other hand, highly acetylated p53 cannot be degradated by MDM2 in the DNA-damaged cells. Consequently, HDAC inhibitors are considered as candidate drugs for cancer treatment because they can stabilize and activate p53 by maintaining the acetylated state of p53. In prostate cancer cells, trichostatin A (TSA) and CG-1521 stabilize acetylation of p53 at Lys382 and Lys373, respectively. Although TSA induces only cell cycle arrest, CG-1521, in addition to cell cycle arrest, induces mitochondrial apoptotic pathway activation (Roy et al., 2005). The reason why these inhibitors differentially stabilize acetylations or induce different outcomes remains unclear. However, they could be powerful tools for cancer treatment.

Ubiquitination

Ubiquitin/proteasome-dependent protein degradation is important for rapid signal transduction. Protein is targeted to the proteasome modifiers in a series of ubiquitin-related enzymes, i.e., E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin protein ligase). Recognition by the ubiquitin molecules is a hallmark of degradation by the 26S proteasome machinery (Hochstrasser, 1995). It is well established that MDM2 binds to the N-terminal transactivation domain of p53, thereby ubiquitinating p53 at six Lys residues (Lys370, Lys372, Lys373, Lys381, Lys382, and Lys386) located in the p53 C-terminal regulatory domain. MDM2-mediated p53 ubiquitination regulates, not only the stability of the p53, but also p53 subcellular localization (Lohrum et al., 2001). Importantly, MDM2 cellular expression determines whether MDM2 induces p53 destruction or nuclear export (Li et al., 2003). Li et al demonstrated that low levels of MDM2 induce p53 mono-ubiquitination and nuclear export, whereas high levels give rise to p53 poly-ubiquitination and degradation. Besides MDM2, constitutively photomorphogenic 1 (COP1) also negatively regulates p53 levels in unstressed cells (Dornan et al., 2004). After DNA damage, ATM phosphorylates COP1 at Ser387 and triggers its nuclear exclusion (Dornan et al., 2006). Recent study showed that this Ser387 phosphorylation serves as 14-3-3σ binding site and COP1 is translocated with 14-3-3σ into the nucleus, and then degradated (Su et al., 2010). Moreover, p53 stability is also modulated by p53-induced RING-H2 protein (Pirh2) (Leng et al., 2003) and ARF-BP1 (Chen et al., 2005). While many E3 ligases for p53 have been reported, it is not known what is predominant for p53 as cross examination of the interactions among these molecules has not been investigated. However, MDM2-mediated ubiquitination of p53 associated proteins is involved in the decision making of p53 functions. In unstressed cells, two p53 Ser46 pro-apoptotic kinases, DYRK2 and HIPK2, are targeted for MDM2-mediated ubiquitination (Rinaldo et al., 2007; Taira et al., 2010) (Fig. 2). Upon sub-lethal DNA damage, p53 suppress HIPK2 expression by p53-

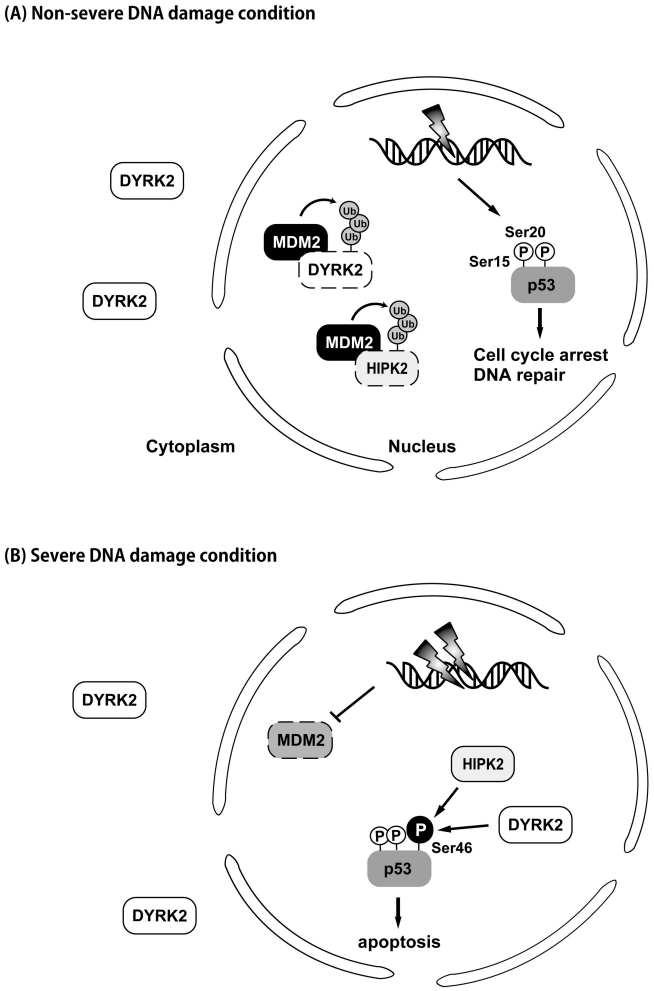

Fig. 2. Models for HIPK2- and DYRK2-mediated Ser46 phosphorylation in response to DNA damage. **A.** Under normal or non-severe DNA damage conditions, nuclear DYRK2 and HIPK2 are ubiquitinated by MDM2, result in decreased their expressions. **B.** Upon exposure to genotoxic stress, attenuation of MDM2 expression and function cause stabilization of HIPK2 and DYRK2 expression, and then HIPK2 and DYRK2 phosphorylate p53 at Ser46 to induce apoptotic cell death.

induced MDM2-mediated degradation. However, exposure to severe cellular damage attenuates MDM2 expression, leading to increased HIPK2 levels, and resulting in p53 phosphorylation at Ser46 (Rinaldo et al., 2007). Our recent study showed that nuclear DYRK2 is constitutively ubiquitinated by MDM2 under normal conditions. In response to DNA damage, ATM phosphorylation of DYRK2 inhibits the interaction of MDM2 with DYRK2, stable DYRK2 expression is thus maintained in the nucleus. Consequently, in unstressed cells, DYRK2 is expressed in the cytoplasm. However, after DNA damage, DYRK2 localizes in both the nucleus and the cytoplasm (Taira et al., 2010). Taken together, ATM exerts, not only inhibition of MDM2-mediated p53 ubiquitination by p53 Ser15 phosphorylation, but also stabilization of p53 regulators that accelerate p53 pro-apoptotic function.

Promoter selectivity of p53

p53 binds to p53-response element (p53-RE) which consists of two consensus DNA binding sequence (5'-PuPuPuC(A/T)(T/A)GPyPyPy-3') and few spacer sequences. Although many p53 target genes that contain p53-REs in the promoter region are identified, the selective transactivation mechanism is not well understood. Recent studies have demonstrated that p53 promoter binding is regulated by post-translational modifications, p53-binding co-factors, and promoter DNA topology of target genes (Menendez et al., 2009). Previous studies demonstrated that p53 Lys120 acetylation and Ser46 phosphorylation induces apoptosis-related gene expression. As Lys120 locates within p53 DNA binding domain, Lys120 is a critical regulating site for p53-DNA interaction. Pan and Nussinov showed that Lys120 modulates p53 promoter recruitment allosterically, which would result in selective co-factor binding at p53-RE regions (Pan and Nussinov, 2010). Contrary to Lys120, Ser46 locates in the N-terminal transactivation domain. Although many reports support that Ser46 is a critical site for the p53-mediated apoptosis induction, it is unclear how Ser46, which locates outside DNA binding domain, regulates selective transactivation of p53 target genes. One possibility is that post-translational modifications change p53 interaction with its co-factors. There are a large number of studies showing that p53 co-factors, including p53BP1, Hzf and hCAS/CSE1L (Iwabuchi et al., 1994; Das et al., 2007; Tanaka et al., 2007), contribute to p53 promoter selectivity. Hzf cooperates with p53 to induce p21 expression. On the other hand, hCAS/CSE1L associates with p53, thereby inducing PIG3 and p53AIP1 expression. However, it remains unclear how these co-factors regulate p53 recruitment to cell cycle arrest- or apoptosis-related gene promoters. Another possibility is that these modifications change the tertiary structure of p53. The C-terminal methylations of p53 by PRMT5 affect p53 tetramerization. Interestingly, the latest study showed that the strength of DNA binding cooperativity via p53 dimer-forming salt bridges within the central DNA-binding domain regulates the promoter affinity toward the consensus motif of p53 (Schlereth et al., 2010). In this regard, the strength of p53 dimer-dimer interaction determines their affinity to promoters of the target genes. Moreover, according to NMR and crystal structural analysis, spacers that intervene between two p53-REs determine promoter affinity/cooperativity of p53 (Pan and Nussinov, 2009). Short spacers (0-2 bps) strengthen p53 dimer-dimer interactions, thereby increasing p53-DNA binding. Long spacers (10bps) make p53-REs assign on the same face of DNA strands toward p53 and thereby p53 can be easily recruited to p53-REs. Meanwhile, spacers with middle length (5bps) allocate p53-REs to the opposite face from p53, which diminishes their cooperativity (Wang et al., 1995). Thus, it is plausible that selectivity of p53 target genes is determined by p53 conformational change, which result from post-translational modification and co-factors binding, on the DNA.

Conclusions and future prospects

Two major functions of p53 is cell cycle arrest and induction of apoptosis, and the balance of these events determines cell fate. Over the past two decades, numerous modifying enzymes for p53 have been identified, and those functional significances revealed. Recently, Loewer and colleagues quantified p53 levels in human living cells by using quantitative time-lapse microscopy (Loewer et al., 2010). They demonstrated that p53 expression increases transiently during normal cell cycle. However, p53 does not induce its target genes, such as p21, during oscillations. On the other hand, sustained severe DNA damage changes the modification state of p53 and then, activated p53 induce p21 expression to arrest cell cycle. Thus, p53 modifications are essential for a quick response to stimuli. In particular, ubiquitination and deacetylation rapidly attenuate p53 function through regulating its expression and function, respectively. Due to the rapid attenuation, cells can escape from p53-mediated cell cycle arrest and re-enter the cell cycle. On the other hand, phosphorylation, acetylation, and methylation widely regulate p53 functions including promoter selectivity and co-factor interaction. Notably, Ser46 phosphorylation and Lys120 acetylation are critical modifications for switching p53 function to pro-apoptotic. These findings suggest that acceleration of Ser46 kinase and Lys120 acetyl transferase activities enable tumor cells to be eliminated. These apoptosis-modulators could be molecular targets for the development of novel cancer therapies. Indeed, expressions of two Ser46 kinases, DYRK2 and HIPK2, are negatively regulated by MDM2. Based on these findings, attenuation of MDM2 activity accelerates the p53 role in apoptosis induction. As described above, phosphorylation, acetylation, and methylation of p53 affect its promoter selectivity, however, how these

modifications directly regulate p53 promoter affinity remains unclear. To further elucidate the role of p53 modifications, topological studies might be required. In recent years, it has been reported that p53 also transactivates some microRNA expressions. Many studies focus on genome wide microRNA profiling, and miR-34 is identified as a direct target of p53 (Chang et al., 2007; He et al., 2007; Raver-Shapira et al., 2007). How microRNA expression is regulated by p53 and which miRNAs are induced by p53 in response to apoptotic stimuli remains elusive. To understand the physiological function of p53, further investigations are needed.

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