Posttranslational Modification of p53: Cooperative Integrators of Function

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The p53 protein is modified by as many as 50 individual posttranslational modifications. Many of these occur in response to genotoxic or nongenotoxic stresses and show interdependence, such that one or more modifications can nucleate subsequent events. This interdependent nature suggests a pathway that operates through multiple cooperative events as opposed to distinct functions for individual, isolated modifications. This concept, supported by recent investigations, which provide exquisite detail as to how various modifications mediate precise protein–protein interactions in a cooperative manner, may explain why knockin mice expressing p53 proteins substituted at one or just a few sites of modification typically show only subtle effects on p53 function. The present article focuses on recent, exciting progress and develops the idea that the impact of modification on p53 function is achieved through collective and integrated events.

INTRODUCTION AND HISTORICAL PERSPECTIVE

The p53 tumor suppressor was shown to be a phosphoprotein (Jenkins et al. 1984; Lane 1992) shortly after its discovery (first as an oncoprotein) 30 years ago by three independent groups, and the first phosphorylated sites, Ser312 and Ser389 in mouse p53, were identified soon thereafter (Samad et al. 1986). These early studies involved analyses by thin-layer chromatography of radioactive peptides of p53 from transformed rodent cells in which p53 was stabilized through its interaction with a DNA tumor virus antigen such as the SV40

T-antigen (Meek and Eckhart 1988) and/or by automated Edman sequence analysis of radiolabeled p53 peptides to infer the site(s) of phosphorylation. In some cases, it could be shown that a known, purified serine/threonine kinase was capable of phosphorylating p53 in vitro, but demonstrating that a specific kinase phosphorylated p53 in vivo was extremely difficult, as was showing that phosphorylation changed the functional properties of p53. Nevertheless, steady progress was made through the late 1980s and early 1990s toward overcoming these hurdles.

Interest in p53 posttranslational modifications (PTMs) increased dramatically in response

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to several reports. A role for p53 in transcription was proposed in 1990 on the basis of studies in yeast, and subsequently p53 was shown to have sequence-specific DNA binding activity (reviewed in Lane 1992). In 1991, Kastan and colleagues (Kastan et al. 1991) showed that p53 protein levels increased in response to the exposure of cells to DNA damage-inducing agents, and that the increase correlated with the inhibition of cell cycle progression. In 1992, Lees-Miller et al. (Lees-Miller et al. 1992) showed that the DNA-activated protein kinase, DNA-PK, phosphorylated p53 in vitro on Ser15 and Ser37 in the amino-terminal transactivation domain. Hupp et al. (Hupp et al. 1992) subsequently proposed an allosteric model for p53 activation as a DNA binding protein because of posttranslational modifications at its carboxyl terminus. Thereafter, it was reported that phosphorylation of Ser15 induced the dissociation of MDM2 from p53, which resulted in p53 stabilization (Shieh et al. 1997). These were the first hypothesized functional roles for p53 PTMs, and, whereas we now know that the mechanisms are more complex (as described later), the suggestions from these early studies were important in establishing a strong interest in characterizing p53 PTMs.

The development of site-specific antibodies that recognized p53 only when modified at a specific site greatly simplified the analysis of PTMs and led to rapid advances. More recently, mass spectrometry has facilitated the identification of additional PTMs, and the catalog of p53 PTMs now comprises modifications to approximately 50 sites throughout the polypeptide and includes phosphorylation, acetylation, mono- and di-methylation, glycosylation, ubiquitylation, neddylation, sumoylation, and poly-ribosylation (summarized in Fig. 1). The catalog of p53 PTMs has been extensively reviewed (Meek 1999; Appella and Anderson 2001; Bode and Dong 2004; Toledo and Wahl 2006; Olsson et al. 2007; Anderson and Appella 2009) and now is possibly nearly complete. In most cases, however, the functional roles of these modifications and knowledge of the signaling pathways that lead to them are far from well understood.

The purpose of the present article is to focus on selected recent advancements that have elevated our understanding of how these modifications function mechanistically to regulate key events in p53 biology. Accordingly, we identify four aspects of recent research that are at the forefront of this area. These are: (1) the cooperative role of amino-terminal phosphorylation events in regulating interactions of p53 with the p53 inhibitor MDM2 or the transcriptional coactivator proteins p300 (KAT3B) and CBP (KAT3A); (2) the function of growth factor-mediated phosphorylation in coordinating physiological and developmental signals; (3) the critical involvement of acetylation events in selectively stimulating p53-dependent transactivation; and (4) the growing awareness of the importance of demodifying enzymes in providing acutely sensitive mechanisms for controlling p53 function. These studies highlight a striking level of complexity, cooperation, coordination, integration, and specificity underpinning a rapid, reversible, and highly sensitive system that is exquisitely selective in meeting the needs of the cell according to the type and intensity of the stimuli that bear on its biological status.

THE SEQUENTIAL AND INTERDEPENDENT CHARACTER OF p53 POSTTRANSLATIONAL MODIFICATIONS

Many phosphorylation events on p53 are stimulated by a variety of genotoxic and nongenotoxic agents (Anderson and Appella 2009), but there are significant differences in the degree of modification of different residues achieved using different stress-inducing agents (for example, see Saito et al. 2003). There is little evidence for any phosphorylation of p53 following induction through the ARF pathway (de Stanchina et al. 1998), although acetylation of p53 has been reported during this event (Ito et al. 2001; Mellert et al. 2007). Strikingly, the phosphorylation of Ser15 (mediated principally by the ATM and ATR protein kinases in response to genotoxic stress) acts as a nucleation event that promotes or permits subsequent sequential modification of many

Figure 1. Posttranslational modification of human p53. The figure is updated from Anderson and Appella (Anderson and Appella 2009). The principal functional domains of p53 are shown together with the sites of modification and their potential modifying and demodifying enzymes.

residues (Sakaguchi et al. 1998; Sakaguchi et al. 2000; Saito et al. 2002; Saito et al. 2003), and cells lacking functional ATM fail to modify efficiently serines 9, 15, 20, and 46 in particular (Saito et al. 2002). Additionally and importantly, ATM-initiated phosphorylation promotes the recruitment of histone/lysine acetyltransferases (HAT), such as p300 and CBP (Lambert et al. 1998; Dumaz and Meek 1999; Dornan and Hupp 2001; Polley et al. 2008; Feng et al. 2009; Jenkins et al. 2009; Lee et al. 2009a; Teufel et al. 2009), leading to acetylation of multiple lysine residues in the DNA binding (DBD) and carboxy-terminal domains of p53. These modifications are thought to contribute to the stabilization of p53 by blocking ubiquitylation (Sakaguchi et al. 1998; Ito et al. 2002), or to modulating binding of p53 to specific response elements (detailed later).

Ser15 is also phosphorylated through the AMPK (AMP-activated protein kinase) pathway in response to glucose depletion and mediates a p53-dependent metabolic arrest at G1/S (Jones et al. 2005), although it is unclear whether AMPK itself modifies Ser15 directly. Nevertheless, this is a milestone discovery demonstrating that Ser15 phosphorylation is a critical focal point for cellular stresses that are independent of DNA damage.

THE INDUCTION AND ACTIVATION OF p53

p53 is rapidly turned over in normal unstressed cells mainly through the action of MDM2, a RING finger type E3 ligase that promotes the poly-ubiquitylation and proteasomal degradation of p53, and additionally inhibits p53-mediated transactivation. MDM2 is critical for maintaining p53 levels, both in developing

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and in adult mice (Jones et al. 1995; Montes de Oca Luna et al. 1995; Ringshausen et al. 2006). However, several other p53-targeted ubiquitin ligases, including Pirh2, COP1, CHIP, ARF-BP1, E6-AP, TOPORS, TRIM24 (Allton et al. 2009), and MKRN1 (Lee et al. 2009b) also contribute to p53 turnover (Brooks and Gu 2006). The main p53 targets of MDM2-mediated ubiquitylation are the six carboxy-terminal lysines (K370, K372, K373, K381, K382, and K386) (Rodriguez et al. 2000).

Induction of p53 involves uncoupling it from its negative regulators, principally MDM2 and the related protein MDM4, which, like MDM2, inhibits p53-mediated transactivation (Marine et al. 2007). Posttranslational modification of p53 plays a key role in this process, at least in the context of the DNA damage response (see later). A complementary, but not mutually exclusive, model is that MDM2 itself is a key target of the various stress signaling pathways that impinge on p53. Thus, inhibition and/or rapid degradation of MDM2 and MDM4 have also been proposed to lead to a rapid accumulation of p53 and activation of its transcription functions (reviewed in Toledo and Wahl 2006; Meek 2009).

FUNCTIONAL AND SYNERGISTIC ROLE OF AMINO-TERMINAL PHOSPHORYLATION IN REGULATING ASSOCIATION WITH p300/CBP AND MDM2

The closely related transcriptional coactivator proteins p300 and CBP each assume a dual role in regulating p53 function. On the one hand, they promote the ubiquitylation and turnover of p53 by MDM2 (Grossman et al. 1998; Grossman et al. 2003) while having critical roles in mediating p53-dependent transactivation in response to genotoxic stress (Avantaggiati et al. 1997; Gu et al. 1997; Lill et al. 1997), and both act to modify histones and open up promoter regions to the core transcriptional apparatus (Espinosa and Emerson 2001). They also inhibit p53 degradation by acetylating lysine residues in the carboxyl terminus of p53 that are normally targets for ubiquitylation. p300 and CBP contain multiple

domains including regions important for protein–protein interaction and HAT activity. p53 binds to each of four structurally similar domains within p300/CBP: Taz1, Kix, Taz2, and IbiD (Avantaggiati et al. 1997; Gu et al. 1997; Grossman et al. 1998; Van Orden et al. 1999; Livengood et al. 2002; Teufel et al. 2007). Indeed, Teufel et al. proposed a “wrap-around” model wherein each of these four p53-binding domains in the p300 monomer interacts with an individual subunit of the p53 tetramer (Teufel et al. 2007) (Fig. 2).

The transactivation domain of p53 falls into two distinct structurally similar subdomains termed TAD1 (residues 1–40) and TAD2 (41–83) (Fig. 1). Although these domains each bind independently to p300/CBP to mediate transcription (Chang et al. 1995; Candau et al. 1997), they bind simultaneously to distinct interacting surfaces within a single p300/CBP domain (for example, KIX [Lee et al. 2009a]) and act synergistically within the context of the full length p53 molecule (Fig. 3) (Teufel et al. 2007; Ferreon et al. 2009; Lee et al. 2009a; Teufel et al. 2009). Additionally, TAD2 forms a relatively tighter association with the Taz2 domain than does TAD1 (Ferreon et al. 2009). This arrangement supports formation

Figure 2. Multisite phosphorylation of the amino terminus of p53 regulates the interaction with MDM2 and with p300/CBP. “Wrap around” model proposed by Fersht and colleagues (Teufel et al. 2007) for the simultaneous binding of one monomer of p300/CBP to tetrameric p53. These interactions are stimulated by multisite phosphorylation of the amino terminus of p53.

Figure 3. Mutually exclusive binding of MDM2 and p300/CBP to p53. Four similar subdomains of p300 (Taz1, Taz2, Kix, and IBiD) can each simultaneously bind to the TAD1 and TAD2 transactivation subdomains of p53. Binding to TAD2 is essentially phosphorylation-independent but multisite phosphorylation within TAD1 greatly stimulates its interaction with p300 but blocks association with MDM2. p300 acts as a scaffold in mediating MDM2-dependent ubiquitylation and degradation of p53: Concurrent binding of MDM2 to TAD1 and p300 to TAD2 may facilitate p53 turnover.

of a ternary complex in which TAD2 interacts with one of the p300/CBP domains, whereas TAD1 from the same p53 molecule simultaneously interacts with MDM2. This ternary complex is thought to promote ubiquitylation and degradation of p53 and fits with earlier data highlighting a role for p300 in this process (Grossman et al. 1998; Grossman et al. 2003). In this model, the ability of MDM2 and TAD1 to compete for binding to p300/CBP determines whether the outcome will be p53 degradation assisted by p300/CBP or the assembly of active transcriptional complexes.

Given these two seemingly contradictory roles for p300/CBP, how might these be resolved? Several studies have now provided compelling evidence that multisite amino-terminal phosphorylation of p53 has a profound influence on the interaction of p53 with p300/CBP and may mediate the switch between ternary complex formation with MDM2 and full association with p300/CBP. Earlier studies revealed that phosphorylation of amino-terminal sites in p53, including Ser15, Thr18, and Ser20, increases the association of p53 with p300/CBP and stimulates p53 transactivation function (Lambert et al. 1998; Dumaz and Meek 1999; Dornan et al.

2003; Finlan and Hupp 2004). Additionally, phosphorylation of these residues was shown to block MDM2 binding and to lead to decreased turnover of p53 (Shieh et al. 1997; Böttger et al. 1999; Chehab et al. 1999; Craig et al. 1999; Dumaz et al. 1999; Unger et al. 1999; Sakaguchi et al. 2000; Dumaz et al. 2001; Schon et al. 2002). These studies suggested a dual role whereby amino-terminal phosphorylation could act as a *switch* by simultaneously promoting p53-mediated transactivation and uncoupling its inhibition by MDM2. Recently, biophysical analyses, using a range of phosphorylated amino-terminal peptides, have shown that phosphorylation of individual residues (Ser15, -20, -33, -37, -46, -55, and Thr18) stimulates, to various degrees, the interaction of these with each of the four p53-binding sites on p300/CBP (Polley et al. 2008; Feng et al. 2009; Ferreón et al. 2009; Jenkins et al. 2009; Lee et al. 2009a; Teufel et al. 2009). All authors agree that, among these residues, Thr18 phosphorylation has the most potent impact. Moreover, di- or multiple phosphorylation events can act cooperatively, increasing the p53/p300 interaction by as much as 80-fold, although the extent to which this occurs varies among the different studies. Additionally, the association of TAD1 with MDM2 is significantly weakened by phosphorylation of Thr18 (Ferreón et al. 2009; Teufel et al. 2009). Interestingly, although other amino-terminal phosphorylation events have little influence on MDM2 binding individually, phosphorylation of all seven amino-terminal sites inhibits such binding to MDM2 by four times as much as does phosphorylation of Thr18 alone (Teufel et al. 2009). This evidence supports a mechanism whereby differential phosphorylation events mediated by differences in the type and intensity of the stress, and the cell type, not only fine-tune the affinity of the p53-p300/CBP interaction and consequently the transcriptional outcome but also collectively constitute a potent regulatory switch favoring TAD1 binding to p300/CBP over its interaction with MDM2 (Fig. 4).

In addition to these biophysical analyses, resolution of the structure of the Taz2 domain

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Figure 4. Sequential multiple modifications may act as a switch that excludes MDM2 binding in favor of interaction with transcriptional proteins. Multisite phosphorylation of the amino terminus of p53 blocks the interaction with MDM2 and favors recruitment of the HATs p300 and CBP that subsequently can acetylate numerous lysine residues in p53, further adding to the exclusion of MDM2 and favoring the engagement of transcriptional components.

in complex with the TAD1 peptide of p53 has provided significant insight into the molecular interactions mediating p53-p300 association and suggests that phosphorylation of Ser15 and particularly of Thr18 increases electrostatic interactions with specific arginine residues in the Taz2 domain, thereby strengthening the interaction (Feng et al. 2009).

Although phosphorylation events within the TAD1 domain can clearly mediate p53-Mdm2-p300/CBP interactions, phosphorylation of sites within TAD2 seems to have little effect on p300 binding (Jenkins et al. 2009; Teufel et al. 2009). It is likely, however, that these modifications play a different role in p53-mediated transactivation. The TAD2 region of p53 also interacts with other proteins, such as the p62 subunit of the general transcription factor TFIIF, an interaction that is important for p53-stimulated transcriptional events following relaxation of the chromatin structure within the promoter region. Phosphorylation of Ser46, a target of p38 MAPK (Bulavin et al. 1999; Perfettini et al. 2005), HIPK2 (D'Orazi et al. 2002; Hofmann et al. 2002; Möller et al. 2003; Dauth et al. 2007), DYRK2 (Taira et al. 2007), and possibly other kinases, and of Thr55, which

is phosphorylated by the TAF1 (TAF_{II}250) transcription factor (Li et al. 2004), each stimulate the interaction of TAD2 with the pleckstrin homology (PH) region of p62 (Di Lello et al. 2006). Additionally, p53 competes with the α subunit of TFIIE for a common binding site in p62, and phosphorylation of Ser46 and Thr55 additively favors p53 binding (Di Lello et al. 2008). Phosphorylation of these residues may therefore govern transcriptional outcome by dynamically influencing whether p62 preferentially interacts with TFIIE or p53. Phosphorylation of p53 is also likely to regulate interaction with other transcription modulators such as PC4 (Rajagopalan et al. 2009) or SMAR1 (Pavithra et al. 2009), but much work is needed to characterize fully the influence of phosphorylation on these interactions.

PHOSPHORYLATION OF SERINES 6 AND 9 – DEVELOPMENTAL AND CANCER-RELATED ROLES

Serines 6 and 9 were originally identified as targets of the protein kinase CK1 family members, CK1 δ and CK1 ϵ (Knippschild et al. 1997; Higashimoto et al. 2000). These sites are

modified after exposure to various genotoxic and nongenotoxic agents (Higashimoto et al. 2000; Saito et al. 2003), but it is unclear what functional role(s) these two modifications have in the DNA damage/stress response. Strikingly, they assume a critical role in the context of mesoderm development in *Xenopus* (Cordenonsi et al. 2007) (Fig. 5). p53 is required for efficient induction of mesoderm-specific gene expression and integrates signaling events downstream of transforming growth factor- β (TGF- β) and fibroblast growth factor (FGF). Mechanistically, this integration necessitates p53 interacting with TGF- β -activated Smad2 and is mediated through the phosphorylation of Ser6 and Ser9. p53 proteins with alanine residues substituted at these phosphorylation sites fail to interact with Smad2 and show impaired mesoderm-inducing ability in *Xenopus* embryos. Additionally, p53 expression restores the TGF- β cytostatic program to human H1299 cells through increased p21 expression. Notably, however, S6A or S9A mutants cannot mediate TGF- β -dependent p21 expression or growth arrest but do fully retain the ability to transactivate expression of other p53-responsive

genes such as MDM2 and BAX. These findings highlight a critical specificity in the function of these modifications. The protein kinases CK1 δ and CK1 ϵ are important for the phosphorylation of p53 in response to FGF signaling, but the mechanism by which the Ras/MAPK pathway promotes CK1-mediated phosphorylation of p53 is not apparent.

The phosphorylation of Ser6 and Ser9 may also be important in tumorigenesis and metastatic progression promoted by TGF- β , activated Ras, and mutant p53 (Adorno et al. 2009). According to this model, Ras signaling promotes the phosphorylation of mutant p53 and leads to the formation of a mutant p53/Smad complex that can sequester p63, a p53 family member, thereby inhibiting its ability to activate expression of key antimetastasis genes. The model explains why TGF- β , which normally is cytostatic, has prometastatic properties in tumor cells expressing activated Ras and mutant p53. It additionally predicts that antitumor drugs, by inducing the phosphorylation of Ser6 and Ser9, may actually promote aggressive tumorigenesis; however, this idea remains to be tested.

Figure 5. Phosphorylation and function of serines 6 and 9 in p53. Phosphorylation of Ser6 and Ser9 in p53 integrates TGF- β - and FGF-signaling by promoting the interaction of p53 with Smad2. These residues are also modified in response to various genotoxic and nongenotoxic stresses, but their function in this context is not yet defined.

OTHER PHOSPHORYLATION EVENTS IN p53

Owing to space constraints, the roles of other phosphorylation sites in p53 will not be discussed here, as they have been covered comprehensively in several reviews (Meek 1999; Appella and Anderson 2001; Bode and Dong 2004; Toledo and Wahl 2006; Olsson et al. 2007; Anderson and Appella 2009).

CRITICAL ROLE OF ACETYLATION IN SELECTIVELY REGULATING p53 FUNCTION

Many of the lysines targeted by ubiquitylation (and other modifications described later) are also acetylated. Importantly, ubiquitylation and acetylation (as well as neddylation and methylation) are mutually exclusive events with different outcomes for p53 regulation.

Seven carboxy-terminal lysine residues (K305, K370, K372, K373, K381, K382, and K386) and one DBD residue (K164) are acetylated by

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CBP (KAT3A)/p300 (KAT3B) (Gu and Roeder 1997; Sakaguchi et al. 1998; Liu et al. 1999; Wang et al. 2003; Tang et al. 2008), whereas K320 is acetylated by PCAF (KAT2B) (Sakaguchi et al. 1998; Liu et al. 1999). Acetylation of lysine residues is induced in response to various forms of genotoxic and nongenotoxic stress with the outcome that p53 is stabilized and activated, in part because the acetylated residues cannot be ubiquitinated by MDM2 (Sakaguchi et al. 1998; Ito et al. 2001; Li et al. 2002b). In addition to these residues, K120 is acetylated through the action of Tip60/hMOF, a MYST family HAT that is unrelated to p300/CBP or PCAF (Berns et al. 2004; Sykes et al. 2006; Tang et al. 2006). Interestingly, this site is a DNA contact residue within the DNA binding domain and is a recurrent target for mutation during tumor development. K120 acetylation is induced by DNA damage, and the modified p53 localizes preferentially to the promoters of key proapoptotic genes but not to those involved in cell cycle arrest. Consistent with these observations, a K120R mutant shows no effect on *p21* expression and retains its ability to arrest growth but fails to induce apoptosis (Sykes et al. 2006; Tang et al. 2006). These data suggest that K120 acetylation may have a decisive function in determining the outcome of p53 induction. Interestingly, K120-acetylated p53 is enriched at mitochondria where it is thought to have a transcription-independent function in regulating apoptosis by influencing the BAK/MCL-1 interaction (Sykes et al. 2009). Two additional acetylation sites, K319 and K357, were recently identified in p53 from SV40-transformed monkey (COS-1) cells, but their significance is unknown (Joubel et al. 2009).

An understanding of the roles played by these acetylated residues has been approached using knockin mice in which lysine to arginine substitutions were made at the appropriate sites in p53. Mice substituted at six (Feng et al. 2005) or seven (Krummel et al. 2005) of these sites develop normally and show no increased susceptibility to cancer. Nevertheless, they do show subtle differences in the behavior of p53 in some cell types but not in others, suggesting that acetylation is context-dependent or other

mechanisms can compensate for it. In a more recent study, Tang and colleagues (Tang et al. 2008) eliminated most of the major targets for acetylation (with the exception of K320) and determined the effects of these changes by expressing the mutant p53 proteins at physiological levels in cultured cells. Consistent with data from the knockin mice, substitution of individual lysines or groups of lysines had little or only subtle effects on p53-dependent expression of *p21* (*CDKN1A*), *BAX* (*BAX*), *PUMA* (*BBC3*), and *PIG3* (*TP53I3*). Strikingly, however, p53 in which all eight lysine residues were substituted (8KR) failed to induce *p21* expression, yet was still fully competent to mediate expression of MDM2. These effects on transactivation were reflected in the loss of the ability of the 8KR mutant to mediate cell cycle arrest. The results suggest that: (a) acetylation plays a vital role in p53-mediated cell fate but does not influence the p53-MDM2 negative feedback regulatory loop; (b) the mechanisms of transactivation by p53 (as reflected by the requirement for posttranslational modification) differ depending on the promoter; and (c) there is a degree of redundancy between the various acetylation sites so that loss of one or more can be compensated by the remaining presence of others.

Mechanistically, the acetylated lysines are thought to participate in mediating “anti-repression” (Tang et al. 2008; Kruse and Gu 2009). In this model, p53 situated on the promoters of responsive genes is inhibited from interacting with the transcriptional apparatus through complex formation with MDM2 and MDM4. Acetylation of p53 inhibits its interaction with MDM2 and MDM4. Moreover, unlike wild-type p53, the interaction of the 8KR mutant p53 with MDM2 and MDM4 on cellular promoters such as *p21* or *PIG3* cannot be competitively disrupted by increased expression of the HATs CBP or Tip60. These findings suggest a fundamental role for acetylation that may have been missed in the two existing lysine substitution mouse models (Feng et al. 2005; Krummel et al. 2005) owing to the fact that not all of the acetylation sites had been identified when these animals were generated.

Thus, the question remains whether acetylation is truly indispensable for p53 function until it is resolved with experiments in mice expressing a p53 substitution mutant equivalent to the human 8KR mutant.

OTHER MODIFICATIONS ON LYSINE RESIDUES

p53 undergoes several other modifications that occur on lysine residues, including mono-ubiquitylation (Li et al. 2003; Nie et al. 2007; Carter and Vousden 2008), conjugation of ubiquitin chains through lysine 63 (K63) of ubiquitin (Laine et al. 2006), sumoylation by SUMO-1 and SUMO-2/3 (Gostissa et al. 1999; Rodriguez et al. 1999; Kahyo et al. 2001; Schmidt and Müller 2002; Weger et al. 2005; Bischof et al. 2006), and neddylation (NEDD8) (Xirodimas et al. 2004; Abida et al. 2007; Carter and Vousden 2008). These have recently been discussed in detail elsewhere (Anderson and Appella 2009; Carter and Vousden 2009) and will not be developed further here.

Methylations of lysine and arginine were also recently established as reversible mechanisms that regulate p53 function (reviewed in Scoumanne and Chen 2008). In short, methylation occurs at carboxy-terminal residues K370, K372, and K382 (Fig. 1) (which are also targets for ubiquitylation and acetylation) and can enhance (Huang et al. 2007; Ivanov et al. 2007; Kurash et al. 2008) or suppress (Shi et al. 2007) p53 function depending on the site modified. Lysine methylation occurs in response to DNA damage (Huang et al. 2006; Ivanov et al. 2007; Kurash et al. 2008) and can facilitate (Ivanov et al. 2007; Kurash et al. 2008) or even inhibit (Huang et al. 2006) subsequent acetylation of other residues, a finding that underscores the interactive nature of PTMs in p53. Recently, Jansson et al. reported that three arginine residues in the p53 oligomerization domain (TET), R333, R335, and R337, are symmetrically dimethylated by PRMT5, a Class II methyltransferase (Jansson et al. 2008). These modifications add a significant level of complexity and interplay with other PTMs.

OTHER MODIFICATIONS

Modifications such as the addition of *O*-linked *N*-acetylglucosamine, ADP-ribosylation, prolyl isomerization, and oxidation of methionine have been reported to regulate p53 function. However, they are less well characterized, and their *in vivo* significance is poorly understood at present (Kruse and Gu 2008; Kruse and Gu 2009).

BACK TO THE FUTURE—PTM REVERSAL

All p53 PTMs are potentially reversible, and cellular enzymes have been identified that can reverse several of the PTMs discussed previously. Although these enzymes are less well studied than the transferases and ligases that act on p53, at least four protein phosphatases capable of dephosphorylating specific p53 sites *in vitro* have been identified (Fig. 1). Likewise, several deacetylases (HDACs) and the deubiquitinating enzyme HAUSP were shown to use p53 as a substrate and, through knockout or knock-down experiments, to affect cellular responses to DNA damage and other stresses (reviewed in Bode and Dong 2004; Brooks and Gu 2006). Also, one demethylase, KDM1, which demethylates Lys370, thus preventing binding of the p53 coactivator 53BP1, has been reported to date (Huang et al. 2007).

PTM-removal enzymes may play important roles in the recovery from stress. They may also make significant contributions toward setting the threshold for p53 activation, thereby preventing inappropriate p53 activation. Moreover, given that some modifications are mutually exclusive, de-modifying enzymes may be required to permit modification status to change, particularly in the context of chromatin-bound p53, where it is actively carrying out its function. For example, methylation of p53 at K372 by KMT5 prevents methylation of K370 by KMT3C by blocking the interaction of KMT3C with p53. The level of methylated K372 increases very rapidly in response to DNA damage (Huang et al. 2006); therefore, methylation of K370 by KMT3C, which represses p53-mediated transcriptional activation, could require removal of

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the K372 methyl group to restore p53 activity to its basal level after DNA damage repair. Additionally, bearing these aspects in mind, the demodifying enzymes may influence tumor development and consequently may deserve equal consideration with modifying enzymes as potential targets for novel antitumor drugs. We highlight two examples that underpin these important concepts.

Phosphatases are the best studied of the enzymes that remove PTMs from p53 (Fig. 1). Dephosphorylation of p53 by protein phosphatase 1 (PP1) and PP2A was recently summarized (Anderson and Appella 2009). PPM1D (WIP1), a relatively newly discovered member of the PP2C subfamily, is the product of a p53-responsive gene that not only partially inactivates the p38 and JNK MAP kinases (Fiscella et al. 1997; Takekawa et al. 2000) but also attenuates the p53 and DNA response pathways by dephosphorylating p53-Ser15, MDM2-Ser395 (also an ATM target) (Lu et al. 2007), and PIKK kinases, including ATM itself (Yamaguchi et al. 2007; reviewed in Lu et al. 2008). Analyses using *PPM1D* knockout mice confirm this role and provide evidence that PPM1D also functions in setting the threshold for p53 activation (Shreeram et al. 2006a; Shreeram et al. 2006b). Additionally, PPM1D is overexpressed in 15–18 percent of primary human breast cancers (and several others) and thus functions as an oncogene, whereas inhibition of PPM1D activity or deletion of the gene protects mice from tumors in several model systems (Bulavin et al. 2002; Li et al. 2002a). This demodifying enzyme, when overexpressed, is highly likely to contribute to the development of disease and thus may be a potential drug target.

Four classes of enzymes that reverse protein acetylation have been described based on their homology with yeast HDACs (Yang and Seto 2008). Various groups (Ito et al. 2002; Glozak et al. 2005) have shown that HDAC1, a member of the class I sub-family can deacetylate most, if not all, acetylated p53 residues in vitro and in cultured cells. Interestingly, HDAC1 recruitment is mediated by MDM2 and promotes p53 degradation (Ito et al. 2002). This

activity is not displayed by other class I family members including HDAC-2 and -3, nor by the class II members HDAC-4 and -5.

p53 can also be deacetylated by the class III HDACs, which are homologs of yeast Sir2 and known as sirtuins in mammals. Sirtuins require NAD⁺ as a cofactor and are not inhibited by trichostatin A (TSA). Three groups of researchers showed that SIRT1, the closest homolog to Sir2, interacted with p53 in the nucleus and specifically deacetylated K382 of p53 (Smith 2002). SIRT1-mediated deacetylation prevented p53-dependent transactivation of *CDKN1A* (*p21^{Waf1}* gene), and its activity, which is expressed predominantly in the developing nervous system, can be modulated positively or negatively by a number of cofactors, including necdin, which recruits SIRT1 to the transactivation domain of p53 (Hasegawa and Yoshikawa 2008). Similar to HDAC1, it is likely that SIRT1 can deacetylate all of the major p53 acetylation sites (Brooks and Gu 2009), but supporting data have not yet been published. Of particular interest, SirT1 and SirT2 were identified as the targets of Tenovin-6, a novel and highly potent small-molecule activator of the p53 pathway that not only shows striking cytotoxicity in cultured cells but also inhibits the growth of highly aggressive melanoma xenograft tumors (Lain et al. 2008). These data strongly support the principle that enormous therapeutic opportunities lie in manipulating the activity of key p53-targeted demodifying enzymes.

CONCLUSIONS AND PERSPECTIVES

Never in the field of molecular oncology have so many sites of posttranslational modification in one protein (p53) been modified by so many different enzymes! How do we make sense of such an eye-watering number of PTMs within a single protein?

An idea emerging from recent studies is that emphasis on individual sites of modification and efforts to assign function in a cellular context may be in many cases inappropriate. Although this approach has occasionally been successful, the individual modifications so far

tested in the context of knockin mice have not revealed the striking phenotypes predicted by biochemistry and cultured cell analyses, and the subtle effects observed are often tissue- or cell-type-specific (Hoogervorst et al. 2005; Toledo and Wahl 2006; Armata et al. 2007; Iwakuma and Lozano 2007). This concept is pitched against the assumption that there has been strong selective pressure during evolution to put these modifications in place.

So how do we rationalize this concept? Three potentially interesting themes emerge. First, when considering the structural and biophysical data that have been published recently on the amino-terminal phosphorylation sites, it seems that contact with transcriptional proteins improves as the number of sites phosphorylated increases. Could this finding imply that phosphorylation works best in a collective sense? In other words, that physiological, multi-site modification is the more effective means of switching on a pathway? Considering that Ser15 phosphorylation seems to act as a nucleation event that stimulates modifications of other sites (phosphorylation and acetylation), fine-tuning the outcome of p53 activation would then depend on the extent to which individual signaling pathways targeting individual sites contribute to this whole.

A second emerging theme is the essential yet flexible nature of acetylation. As discussed previously, Wei Gu's laboratory (Tang et al. 2008) showed that p53 that cannot be acetylated is essentially inactive other than as a stimulator of its own degradation apparatus. Having so many acetylation sites could therefore be a safeguard that p53 needs to maintain its function. Nevertheless, we still do not know how acetylation of different residues governs precise protein-protein interactions or the molecular basis for their contribution to promoter selectivity.

A third emerging theme is the interdependence and the sequential nature of these modifications. A principal purpose of amino-terminal phosphorylation is to recruit factors to stimulate transcription; thus, there is a clear integration of multiple sequential events in which phosphorylation-dependent recruitment

of HATs not only opens up the chromatin but contributes further to the activation of p53. Moreover, the "switch" is not a simple one. For example, alone, phosphorylation at the amino terminus of p53 disrupts its interaction with the MDM2 amino-terminal pocket, but there are still other points of contact that are relieved by subsequent acetylation of the carboxyl terminus. Notably, these same modifications simultaneously activate transcription. It is very difficult to see how such complexity could be dissected in a knockin mouse with one or a few substitutions!

Our progress in the study of p53 PTMs has raised a number of key questions. Are any p53 PTMs (e.g., acetylation, [Tang et al. 2008]) truly essential? Are PTMs truly connected with tumor suppression? Data from phosphorylation-site-substituted mice show an influence on tumor susceptibility, albeit subtly and in tissue/cell-specific contexts (Bruins et al. 2004; MacPherson et al. 2004; Sluss et al. 2004; Hoogervorst et al. 2005; Chao et al. 2006; Armata et al. 2007). How susceptible to tumor development would a mouse be if Thr21 (equivalent to human Thr18), or indeed all of the major amino-terminal phosphorylation sites involved in regulating its interaction with MDM2 and p300, were eliminated? And if PTMs do not play a key role in tumor suppression, then is it possible that they regulate one or more of the newly discovered homeostatic functions now attributed to p53? The recent work from Piccolo's group on the interaction with Smad proteins tends to support this idea (Cordenonsi et al. 2007). Moreover, given that p53 represses the expression of many important genes, what role do modifications play in p53-mediated repression? And if, as many studies suggest, phosphorylation and acetylation are involved in selectively influencing p53-mediated transcription, what are the mechanisms?

p53 phosphorylation is not required following its induction through the ARF pathway or in response to synthetic inducers such as Nutlins. So how is this possible, and why then do these modifications occur? Why has evolution selected not only for their presence but for such a wide range of possible modifications and

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combinations of events? Certainly, if one looks at the very exquisite structural and biochemical analyses of phosphorylation site function (discussed previously), it is breathtaking that the paradigms based on these analyses do not hold true (so far) in animal models. How do we rationalize this?

We are surely at a very stimulating and exciting stage in the study of p53 modifications. Although 30 years of effort and progress by many laboratories (much of which could not be included in this article) has enhanced our understanding of how these events regulate p53 function, we are still at an early stage. In particular, the recent demonstration of the genetic separation of the DNA damage response and tumor suppression (Efeyan and Serrano 2007) together with the recognition that p53 most likely evolved, at least in mammals, to perform regulatory roles in development, metabolism, and other normal cellular processes (Aranda-Anzaldo and Dent 2007; Danilova et al. 2008; Tedeschi and Di Giovanni 2009; Vousden and Prives 2009) invites the study of PTMs in these processes. The questions raised by these findings may dominate our attention for some time to come; nevertheless, the steady progress achieved in understanding the roles of p53 PTMs suggests that one may justifiably be optimistic that more interesting and telling answers will emerge over the next few years.

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