The tumor suppressor p53 is the most frequently inactivated gene in human cancers. The p53 protein functions as a sequence-specific transcription factor to regulate key cellular processes, including cell-cycle arrest, DNA repair, apoptosis, and senescence in response to stress signals. P53 is maintained at a low level in the cell, but becomes rapidly stabilized and activated in response to DNA damage, hypoxia, hyperproliferation, and other types of cellular stresses. The stability and transcriptional activity of p53 are tightly regulated through multiple post-translational modifications, such as phosphorylation, acetylation, and ubiquitination. Within the past few years, several studies have established that protein methylation is a novel mechanism by which p53 is regulated. Indeed, histone lysine methyltransferases KMT5 (Set9), KMT3C (Smyd2), and KMT5A (Set8) methylate p53 at specific C-terminal lysines. Lysine methylation enhances or suppresses p53 transcriptional activity depending on the methylation site. Furthermore, the lysine-specific demethylase KDM1 (LSD1) mediates p53 demethylation, which prevents p53 interaction with its co-activator 53BP1 to induce apoptosis. Finally, protein arginine methyltransferases CARM1 and PRMT1 are co-activators of p53 involved in the methylation of histones H3 and H4 to facilitate p53-mediated transcription. In response to cellular stresses, the interplay between p53 methylation, demethylation, and other post-translational modifications fine-tunes the activity of p53 to ultimately prevent tumor formation.

**Key words:** p53, Methylation, Methyltransferases, Transcription, Cancer

**Introduction**

The tumor suppressor p53 is a major coordinator of the cellular response to stress signals, such as DNA damage, hypoxia, and hyperproliferation. Upon stabilization, p53 activates numerous genes involved in cell-cycle arrest (p21 and 14-3-3σ), DNA repair (p53R2 and GADD45), apoptosis (IGFBP3, BAX, and PUMA), or the regulation of its own activity (MDM2) (Harms et al., 2004; Helton and Chen, 2007). In addition, p53 is a transcriptional repressor of genes involved in cell proliferation (Myc), cell-cycle progression (ECT2 and cyclinB1), and replication (RECVQ4) (Ho et al., 2005; Imbriano et al., 2005; Sengupta et al., 2005; Scoumanne and Chen, 2006). The important role of p53 as a cellular gatekeeper is further highlighted by the high frequency of spontaneous p53 mutations in human cancers. In addition, p53 germline mutations are responsible for the high incidence of tumors found in Li-Fraumeni syndrome patients (Malkin et al., 1990).

P53 is a multi-domain protein that contains an activation domain 1 (AD1) (residues 1-42), an AD2 (residues 43-92), a DNA binding domain (DBD) (residues 102-292), a nuclear localization signal (residues 292-325), a tetramerization domain (residues 325-363), and a basic domain (BD) (residues 363-393) (Fig. 1). Each domain plays an important role in p53 function (Scoumanne et al., 2005). But, the activation domains at the N-terminus and the basic domain at the C-terminus are the major sites for p53 regulation through post-translational modifications, such as phosphorylation, acetylation, methylation, and ubiquitination (Bode and Dong, 2004).

In unstressed conditions, p53 is maintained at a low level in the cell due to its degradation by the 26S proteasome. The rapid turnover of p53 is mainly mediated by the binding of E3 ubiquitin ligase MDM2 (mouse double minute 2) in p53-AD1 and p53-DBD, which then ubiquitinates the lysines located in the p53-DBD and p53-BD (Honda et al., 1997; Shimizu et al.,
Methylation regulates p53 function

2002). In response to stress signals, various sensor kinases, such as ATM and ATR, are activated. These kinases initiate a signal transduction pathway, which ultimately leads to p53 phosphorylation at multiple sites in p53-AD1. P53 phosphorylation releases MDM2 binding and promotes p53 stabilization (Chen et al., 1995; Bakkenist and Kastan, 2004). It also facilitates the binding of components of the transcriptional machinery (TBP, TAFII31, and TAFII70) and p53 co-activator acetyltransferase KAT3A/B (CBP/p300) to initiate transcription (Avantaggiati et al., 1997). Indeed, KAT3A/B catalyzes p53 acetylation at lysine residues in p53-BD (K373 and K382), which further stabilizes p53 and promotes p53-DNA interaction (Gu and Roeder, 1997; Knights et al., 2006). Furthermore, KAT3A/B mediates the acetylation of histones H3 and H4 to open the chromatin structure and facilitate transcription. In response to cellular stresses, many other post-translational modifications of p53 (neddylation and symoylation) and protein-protein interactions (Sin3A and histone deacetylase HDACs) are important to finely regulate p53 stability and transcriptional activity (Murphy et al., 1999; Basile et al., 2006; Harms and Chen, 2007).

p53 is one of the few transcription factors regulated through protein methylation/demethylation. In this review, we will present a brief overview on protein methylation/demethylation and describe how methyltransferases/demethylase modulate p53 stability and transcriptional activity. We will also discuss the importance of crosstalks between methylation and other post-translational modifications in the regulation of p53 function.

Protein methylation and demethylation

Protein methylation, the transfer of a methyl group to amino-acids, such as lysines, arginines, and histidines, is an essential process for the regulation of gene expression, protein function, and RNA metabolism (Shen et al., 1998; Chen et al., 1999). Methyltransferases catalyze the methylation of proteins by using S-adenosyl-L-methionine (SAM) as methyl donor (Schubert et al., 2003). Methylation generates the methylated substrate and a by-product, S-adenosyl-L-homocysteine, which is then degraded into adenosine and homocysteine by the enzyme S-adenosyl-L-homocysteine hydrolase. Among the many substrates for protein methylation, histones have been the most extensively studied. Indeed, methylation of histones, in combination with other modifications, constitutes the ‘histone code’ that determine chromatin structure and DNA accessibility for replication, repair, and transcription (Strahl and Allis, 2000).

Histones are mainly methylated on lysine and arginine residues in their N-terminal tails by histone lysine methyltransferases (KMTs) and protein arginine methyltransferases (PRMTs), respectively. The KMTs mediate the transfer of up to three methyl groups to the ε-nitrogen of lysine residues and therefore generate mono-, di-, or trimethylated lysines. All KMTs, except one, contain a SET domain important for the binding of SAM and the interaction with the targeted lysine to catalyze the methyl transfer (Cheng et al., 2005). The KMTs catalyze the methylation of very few non-histone substrates, including p53 and TAF10 (Kouskouti et al., 2004). The PRMTs mediate the transfer of one or two methyl groups to the ω-nitrogen of the guanidinium side chain of arginine. The addition of a second methyl group to monomethylated arginine results in either an asymmetric dimethylarginine (type I PRMTs) or a symmetric dimethylarginine (type II PRMTs). Type I and type II PRMTs can both catalyze the formation of monomethylarginine (Bedford and Richard, 2005). Most of the eleven PRMTs identified to date are type I PRMTs, including PRMT1 and CARM1. Type II PRMTs are PRMT5, PRMT7, and PRMT9.

The methyltransferase activity of PRMT2, PRMT10, and PRMT11 has yet to be determined. The PRMTs are varied in length but share a highly conserved central domain responsible for the methyltransferase activity. Many PRMTs are involved in the methylation of non-histone proteins, such as transcription factors (Stat1) and RNA-binding proteins (hnRNPs and Sm proteins) (Shen et al., 1998).

Methylation was thought for many years to be an enzymatically irreversible modification, until the identification of the first lysine-specific demethylase, KDM1 (LSD1) (Shi et al., 2004). KDM1 is a nuclear amine oxidase, which uses FAD as a cofactor to demethylate mono- and dimethylated lysines (Shi et al., 2004). KDM1-mediated demethylation generates the unmethylated lysine and two byproducts, formaldehyde and H₂O₂. KDM1 specifically demethylates histone H3 at K4 to induce transcriptional repression (Forneris et al., 2005). In addition, KDM1 promotes androgen-receptor-dependent gene activation by demethylating histone H3 at K9 (Metzger et al., 2005). Interestingly, a novel family of proteins, the Jmjc-containing family, possesses histone lysine demethylase activity, with varied specificity for lysine sites and degree of methylation (Tsukada et al., 2006). The identification of KDM1 and other lysine demethylases has recently brought much interest in the search to identify the first arginine demethylase. To date, the protein arginine deiminase 4 (PAD4) is the only enzyme known to convert arginine residues into citrulline but whether this enzyme also mediates deminination of methylated arginine in vivo is still controversial (Wang et al., 2004; Raijmakers et al., 2007).

Regulation of p53 by lysine methyltransferases

The p53 protein contains twenty lysines, six of which are located in the p53-BD. Three of the six lysines in p53-BD are known to be specifically methylated by histone lysine methyltransferases, KMT5 (Set9), KMT3C (Smyd2), and KMT5A (Set8) (Fig. 1) (Allis et
The Set domain-containing protein KMT5 mono-methylates p53 at K372 (Chuikov et al., 2004). KMT5 interacts with p53 through a K/R-S/T-K motif (where K is the K372 substrate), which is conserved in other KMT5 substrates, such as histone H3 and TAF10 (Couture et al., 2006). KMT5 methylation results in the nuclear localization and increased stability of the methylated p53-K372 protein. Furthermore, methylated p53-K372 is transcriptionally hyperactive to induce target genes, including cyclin-dependent kinase inhibitor p21, pro-apoptotic BAX, and MDM2. This ultimately leads to an increase in p53-mediated G2/M arrest and apoptosis. Importantly, the level of methylated p53-K372 protein is increased very rapidly in response to DNA damage. Indeed, DNA damage has no effect on the level of KMT5 protein, but quickly increases KMT5 activity (Ivanov et al., 2007). We may speculate that KMT5 is itself regulated through post-translational modifications, such as phosphorylation and acetylation, in response to stress signals. Nevertheless, more studies are required to determine the mechanisms by which DNA damage affects KMT5 activity.

Lysines in the p53-BD are targets for many post-translational modifications besides methylation, including ubiquitination, acetylation, neddylation, and sumoylation. To this day, the cross-talk between various modifications at a particular site and between modifications at adjacent sites has not been clearly elucidated. In particular, the addition of a methyl group (14 Da in size) to a lysine residue does not change the charge of the residue. However, it may modulate the local hydrophobicity of the p53-BD without altering the overall p53 structure. Indeed, methylation at p53-K372 has no effect on the total ubiquitination of p53 and therefore is unlikely to directly prevent p53 degradation (Nakamura et al., 2000; Ivanov et al., 2007). Interestingly, in a recent study, Ivanov et al. found that the methylation at p53-K372 facilitates the acetylation at p53-K373/K382 by KAT3B. However, the pre-acetylation at p53-K373/K382 prevents the methylation at p53-K372 by KMT5. These findings suggest that p53 methylation at K372 precedes p53 acetylation at adjacent lysines. This is supported by kinetic studies showing that p53 is first methylated and then acetylated when bound to the p21 promoter in vivo (Ivanov et al., 2007). In histone H3, cross-talks between methylation at K4 by KMT5 and acetylation at K9 and K14 by KAT3B are known to activate gene expression (Wang et al., 2001). Indeed, H3-K4 methylation disrupts the binding of NuRD histone deacetylase complex and impairs KMT1A-mediated methylation at H3-K9, a mark of transcriptional repression (Nishioka et al., 2002). Furthermore, KMT5 methylation of TAF10, a component of the general transcriptional machinery, increases its affinity for RNA polymerase II, leading to an increased transcription of TAF10-dependent genes ERA and ERF1 (Kouskouti et al., 2004). Future studies are required to determine whether the release of HDACs, the recruitment of KAT3B, or the recruitment of other co-factors, are involved in the regulation of p53 activity by KMT5-mediated methylation. It is clear however that methylation of p53 by KMT5 is an important and early event in p53 activation in response to cellular stresses.

The p53-BD is methylated in vivo by two additional KMTs, KMT3C and KMT5A. KMT3C, a member of the Smyd (SET/MYND domain) subfamily of SET domain-containing proteins, mono-methylates p53 at K370 (Huang et al., 2006). KMT3C interacts with p53 in vivo, but the binding site has not yet been mapped. Interestingly, the methylation of p53 by KMT3C represses the transcriptional activation of p21 and MDM2 by p53. KMT3C methylation ultimately leads to a decrease in p53-mediated cell-cycle arrest and apoptosis. Furthermore, in response to DNA damage, the level of methylated p53-K370 associated with the p21 promoter is decreased. This suggests that methylation of p53 at K370, in contrast to methylation at K372, has a repressive effect on p53 activity. Similarly, KMT3C is a

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Fig. 1. Schematic representation of the p53 domain-structure and post-translational modifications at the C-terminus. AD: activation domain; DBD: DNA-binding domain; NLS: nuclear localization signal; TD: tetramerization domain; BD: basic domain; Ub: ubiquitination; Ac: acetylation; Me: methylation.
Methylation regulates p53 function

Marker for transcriptional repression in histones. Indeed, the methylation of histone H3 at K36 by KMT3C inhibits transcription in vitro (Brown et al., 2006). KMT3C interacts with two components of the Sin3 histone deacetylase complex, HDAC1 and Sin3A, but whether deacetylase activity is involved in KMT3C-mediated repression remains to be determined. Similarly, the mechanism underlying the repressive effect of methylated p53-K370 needs further investigation. Furthermore, KMT3C expression is not altered in response to DNA damage. KMT3C has no effect on the total level of p53, the level of acetylated p53-K382, phosphorylated p53-S15, or methylated p53-K372. However, the methylation at p53-K372 by KMT5 prevents the methylation at p53-K370 by KMT3C (Huang et al., 2006). This is due to an inhibitory effect of p53-K372 methylation on the interaction between KMT3C and p53. Therefore, it is postulated that, in unstressed conditions, the repressive methylation mark at p53-K370 is prevalent to regulate p53 function. In response to stress signals, the activation of KMT5 and the subsequent methylation of p53 at K372 release the inhibitory methyl mark on p53 to trigger a rapid increase in p53 transcriptional activity. This interplay between p53 methylation sites provides a novel mechanism for the rapid regulation of p53 function in vivo.

The p53 protein is mono-methylated at K382 by a third histone lysine methyltransferase, KMT5A (Shi et al., 2007). Alignments with other KMT5A substrates suggest that KMT5A recognizes a RHRK motif (where K is the K382 substrate) in the p53-BD. KMT5A methylation, similarly to KMT3C methylation, suppresses p53-mediated transcriptional activation of p21 and PUMA. Indeed, the methylation of p53 at K382 prevents p53 binding to p21 and PUMA promoters. This ultimately results in a decrease in p53-mediated apoptosis. Interestingly, the level of methylated p53-K382 is decreased upon treatment with the radiomimetic drug neocarzinostatin, which correlates with an increase in p53-mediated activation of p21 and PUMA. It should be noted that 53BP1 is also a key upstream transducer of the DNA damage response. Indeed, 53BP1 is recruited at sites of double-strand breaks through binding to dimethylated H4-K20 (Botuyan et al., 2006). Subsequently, 53BP1 is involved in the recruitment of p53 and other checkpoint effectors (Chk2 and Brca1) for ATM and ATR-mediated phosphorylation (Wang et al., 2002). However, it is not clear how the binding of 53BP1 to dimethylated p53-K370 facilitates p53 activity and precisely how the methylation state at K370 is regulated in response to cellular stresses.

In summary, lysine methylation brings another level of complexity to the post-translational regulation of p53. Indeed, p53 function is either activated or repressed by lysine methylation, depending on the methylation site, the methylation state, and the status at surrounding residues. It is likely that, in the near future, novel p53 co-factors will be identified that specifically bind to p53 at methylated lysines to regulate its function.

Regulation of p53 by arginine methyltransferases

Most of the twenty-seven arginines in p53 are located in the p53-BDB. These arginines are essential for maintaining the structure of the DBD and the interaction between p53-BDB and the p53 responsive element in the target gene. Indeed, mutations of several arginines in the p53-BDB (R248, R273, R249, and R282) are common ‘hotspots’ in human cancers (Harms and Chen, 2006). Furthermore, the p53 protein possesses arginines in the AD2 (R65 and R72), the nuclear localization signal (R306), the tetramerization domain (R333, R335, R337, R342, and R353), and the basic domain (R379). Interestingly, a common p53 polymorphism is located at codon 72 in AD2, which encodes for an arginine or a proline. Both p53 variants are structurally and functionally distinct, with the R72 variant being more efficient to activate apoptotic gene

Diverse methylation states of histones (mono-, di-, or trimethylation) have specific impacts on binding of histone-interacting proteins. A variety of protein domains can specifically recognize motifs containing a methylated lysine, such as chromo, tudor, and MBT domains (Kim et al., 2006). The three p53 lysine methyltransferases identified so far are only able to mono-methylate p53. However, it is possible that yet unknown methyltransferases mediate the di- or trimethylation of p53. Indeed, p53 is dimethylated at K370 in vivo by an unidentified enzyme (Huang et al., 2007). Interestingly, the level of dimethylated p53-K370 is increased in response to DNA damage at the promoter of p21. This reveals that dimethylation at p53-K370, in contrast to monomethylation by KMT3C, is an activating methyl mark. Interestingly, dimethylated p53-K370 is specifically bound by 53BP1 (p53 binding protein-1) through its tandem Tudor domain (Huang et al., 2007). The interaction between p53 and 53BP1 enhances the transcriptional activation of p21, MDM2, and PUMA by p53. It should be noted that 53BP1 is also a key upstream transducer of the DNA damage response. Indeed, 53BP1 is recruited at sites of double-strand breaks through binding to dimethylated H4-K20 (Botuyan et al., 2006). Subsequently, 53BP1 is involved in the recruitment of p53 and other checkpoint effectors (Chk2 and Brca1) for ATM and ATR-mediated phosphorylation (Wang et al., 2002). However, it is not clear how the binding of 53BP1 to dimethylated p53-K370 facilitates p53 activity and precisely how the methylation state at K370 is regulated in response to cellular stresses.

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PERP and induce apoptosis (Dumont et al., 2003). To date, it remains unclear how the polymorphism at codon 72 modulates p53 transcriptional activity. Interestingly, p53-AD2 is the site for p53 interaction with co-activator KAT3A/B (residues 71-90) and co-repressor deacetylase mSin3/HDAC complex (residues 61-75) (Murphy et al., 1999). Therefore, arginines located in the p53-AD2 (R72 and R65) may provide interesting targets for post-translational regulation by PRMTs.

To date, there is no indication that p53 is arginine methylated. However, p53 interacts with two class I PRMTs in vivo, PRMT1 and CARM1 (An et al., 2004). The p53 protein binds to PRMT1 in the p53-AD1 (residues 1-43) and to CARM1 in the p53-BD (residues 370-393). Both PRMTs function as co-activators of p53 to modulate the methylation status of histones surrounding p53 target genes. Indeed, the ability of p53 to transcriptionally activate GADD45 following UV irradiation depends on the acetylation of histones H3 and H4 by KAT3B, the methylation of H3 by CARM1, and the methylation of H4 by PRMT1. Interestingly, KAT3B and PRMT1 stimulate their respective activity on histones to ultimately boost p53 transcriptional activity. The acetyltransferase activity of KAT3B further stimulates the methylation of histone H3 by CARM1 to enhance p53-mediated transcription. However, the methyltransferase activity of CARM1 is not able to enhance the acetylation of histone H3 by KAT3B (An et al., 2004). These findings suggest that an ordered recruitment and activation of histone acetyltransferases and methyltransferases is required to mediate the essential changes in the chromatin structure associated with p53-dependent transcription. However, it is yet unclear how CARM1 and PRMT1 are recruited by p53 in response to stress signals. In addition, it is still possible that PRMTs methylate p53 or some of its co-factors. Indeed, KAT3A/B is a substrate for arginine methylation by CARM1, but not PRMT1 (Chen et al., 1999). The arginine methylation of KAT3B at specific sites selectively modulates its interaction with co-factors, such as CREB and GRIP1 (Lee et al., 2005; Chen et al., 1999). Therefore, the arginine methylation of p53, KAT3B, or other p53 co-factors may play a significant role in the differential regulation of p53 target genes in response to a particular stress signal.

Regulation of p53 by protein demethylases

Lysine methylation, similarly to other post-translational modifications, is enzymatically reversible. Recently, we and others showed that the lysine-specific demethylase KDM1 is a regulator of the p53-mediated DNA damage response. Indeed, KDM1 interacts with p53 in the nucleus, although the site of KDM1 binding to p53 remains to be mapped. We found that KDM1 knockdown inhibits cell proliferation and delays the initial induction of p53 in response to DNA damage, which leads to decreased activation of p21 and MDM2 (Scoumanne and Chen, 2007). In addition, we found that overexpression of KDM1 has no effect on cell proliferation or p53 transcriptional activity. In contrast, Huang et al. found that the knockdown of KDM1 increases the transcriptional activation of p21 and MDM2 by p53, resulting in an increase in p53-mediated apoptosis (Huang et al., 2007). Furthermore, Huang et al. found that KDM1 mediates the specific demethylation of dimethylated p53-K370, which prevents the binding of p53 co-activator 53BP1. KDM1 is not able to demethylate monomethylated p53 at K370 or K372 in vivo. It would be interesting to explore whether other demethylases, such as members of the JmjC-containing family, are implicated in the demethylation of p53 at some of the KDM1-resistant methylation sites. In addition, it is likely that demethylases are involved in the removal of methyl groups at arginine residues in histones and other proteins. It may be speculated that, similarly to the p53 co-activator function of CARM1 and PRMT1, arginine demethylases may act as co-repressors of p53 function.

Conclusions and future prospects

KMTs, PRMTs, and demethylases are novel regulators of p53 function. Still, several issues need to be addressed in the future. Indeed, the signaling pathways involved in the recruitment of methyltransferases or demethylases to p53 in response to cellular stresses are still unknown. There is also little information on whether the enzymatic activity and specificity of methyltransferases or demethylases are modulated in response to various cellular stresses.

Some controversies have emerged recently on whether lysine modifications at the p53 C-terminus are physiologically relevant. Indeed, homozygous p53(7KR) mutated mice, in which the endogenous p53 is mutated (K to R) at the seven C-terminal lysines, are viable and phenotypically normal (Krummel et al., 2005). In addition, in various cells derived from the p53(7KR) mutated mice, the p53 protein has a normal half-life and only a slight impairment in transcriptional activity. It should however be considered that lysine post-translational modifications, such as methylation, may have both a positive and a negative impact on p53 function. Therefore, lysine mutations may prevent both types of p53 regulation, resulting in an apparently mild phenotype (Shi et al., 2007). It is also possible that by replacing lysines with arginines, artificial sites for protein-protein interactions and possibly arginine methylation are created. Additional in vivo studies are therefore required to decipher the true physiological role of the C-terminal lysines for p53 tumor suppressor function. Interestingly, recent studies suggest that lysines located outside the p53-BD are post-translationally modified (Sykes et al., 2006). Indeed, MDM2 was recently found to ubiquitinate several lysines in the p53-DBD, which results in the destabilization of p53 (Chan et al., 2006). In addition, lysine K120 in the p53-DBD is acetylated by acetyltransferases KAT8 and KAT5 (Sykes
et al., 2006). The acetylated K120-p53 is selectively accumulated at the promoter of PUMA and BAX to mediate p53-dependent apoptosis. It is therefore possible that several lysines or arginines in the DBD or other p53 domains are targeted for methylation.

Changes in histone modifications and chromatin structure play an essential role in the temporal and spatial expression of genes during normal cell growth, development, and differentiation. Similarly, changes in p53 post-translational modifications are important for the timely-regulated expression of specific target genes in response to cellular stresses. By analogy to the 'histone code', a specific pattern of p53 phosphorylation, acetylation, and methylation, is likely to constitute a 'p53 code' that determines whether a specific target gene is activated or repressed in response to a particular signal.

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