Lysine Methylation of the p53 Tumour Suppressor: Analogies with Histone Biology

Genetics

Hayley Porter

Junior Sophister

p53 is an essential mediator of cellular responses to genotoxic stresses. As aberrant p53 activity is highly linked to cancer incidence and oncogenic activity, understanding the regulation of p53 is crucial to our ability to understand and fight many different cancers. Post-translational modifications of p53 mediate its ability to respond to the various genotoxic stresses experienced by cells. These modifications have been found to be strongly related to the post-translational modifications of histone proteins. This review will discuss identified similarities between lysine methylation events on both proteins and how the extent of these similarities can be used to expand our current knowledge surrounding the regulation of p53 activity. Furthermore, formation of a 'p53 code', analogous to the established 'histone code', will be discussed, in which, the versatile nature of p53 modifications, including methylation, has altered consideration of post-translational modifications from 'on/off switch' mechanisms, to highly interactive, fine-tuning mechanisms. The limitations of experimental techniques are important issues to be taken into consideration when evaluating past discoveries and when anticipating future perspectives within the field. Many in vitro systems are subject to aberrant conditions, resulting in abnormal activity of p53. Despite organismal differences, in vivo verification in mouse models or physiologically relevant cell culture environments represents a key method of minimising these shortcomings. Some key p53 modifications still require physiologically relevant in vivo verification, such

as, characterisation of the interaction between p53K382me1 and the L3MBTL1 effector protein.

Introduction

The p53 tumour-suppressor protein acts to protect cells from malignant transformation following exposure to various stresses. This is achieved via p53-dependent induction of either cell cycle arrest or apoptosis. Differential induction of cell cycle arrest genes, such as *p21/CDKN1A*, or pro-apoptotic genes¸ such as *Bax*, *Puma*, and *Noxa*, mediate this decision and result in the distinct cellular outcomes of the specific response pathway (el-Deiry *et al*. 1993, Oda *et al*. 2000, Vousden & Lu 2002). The precise mechanism by which damaged cells choose between these distinct responses, following varied malignancy-inducing stresses, is not yet fully understood, but is generally thought to be mainly influenced by post-translational modifications of p53 (Gu & Roeder 1997). Post-translational modifications of histone proteins are well documented, with each type of modification having been observed to affect chromatin via one of two distinct outcomes. Acetylation¬ and phosphorylation of histone proteins have been recognised as modifiers of the charge density of histone tails, ultimately resulting in physical alteration of chromatin's open/closed state. Contrastingly, methylation, neddylation, sumoylation, and ubiquitination do not physically affect the open/closed state of chromatin, but instead act to modulate interaction with 'reader'/histone-binding proteins, thus controlling the effects of these cellular factors. For example, methylation of lysine 9 on histone H3 (H3K9) has been shown to create a binding site for heterochromatin protein 1 (HP1) family members, allowing these effector proteins to act on chromatin, changing it into its heterochromatin state (Strahl & Allis 2000, Lachner *et al*. 2001). These ideas underlie the 'Histone code' hypothesis, proposed by Strahl and Allis (2000), which brings together the interplay of modifications, combining direct chromatin alterations and modulation of protein-protein interaction to form a powerful mechanism of regulating chromatin structure and hence, transcriptional activity (Jenuwein & Allis 2001). By analogy with the 'histone code', a 'p53 code'/'protein code' has been proposed to explain the functional consequence of

p53 modifications (Toledo & Wahl 2006, Scoumanne & Chen 2008, Sims & Reinberg 2008). This review will explore current knowledge surrounding post-translational modification of p53 by lysine (K) methylation, through the idea of a 'p53 code' of modifications, which regulate the distinct cellular pathways induced by p53 in response to varied forms of stress. Parallels between the 'histone code' and 'p53 code' will be investigated in consideration of potential p53 methylation events, alongside consideration of the effectiveness of current experimental techniques.

Lysine Methylation of p53: How Histone Biology Carved the Way

Similar to the methylation sites of histone H3 and histone H4, p53 has an unstructured terminal tail that contains a number of lysine residues capable of accepting methyl groups. Lysine methylation of histones modulates the surface architecture of the protein, promoting or inhibiting modular binding by effector proteins (West & Gozani 2011). For example, monometylation of histone H4 lysine 20 is seen frequently in condensed chromatin, as this modification allows interaction of the histone with L3MBTL1, a chromatin compaction factor (Kalakonda *et al*. 2008).

Major progress in the field of p53 lysine methylation was made following the discovery of Set7/Set9 methyltransferase (Set7/9) activity towards p53 (Chuikov *et al*. 2004). Methyltranferases are examples of so called 'writer' proteins, which modulate the surface architecture of their substrates via addition of methyl groups. Specifically targeted lysine residues can enter a narrow channel in the core of the Set7/9 protein, where addition of a methyl group is then catalysed (Wilson *et al.* 2002, Kwon *et al*. 2003). Set7/9 was first identified as a methyltransferase with specificity for lysine 4 of histone H3 (Wang *et al*. 2001, Nishioka *et al*. 2002a). Subsequently, lysine residue 372 (K372) of p53 was identified as a Set7/9 target. Comparison with the established Set7/Set9-H3 complex led to the identification of a putative Set7/Set9-p53 complex, with structural and binding similarities helping to verify the p53 methylation event. This methylation event was in fact found to be required for

p53 stabilisation, and although the mechanism of this methylation-induced stabilisation was not established, comparison with mechanisms identified in histone biology allowed hypothesis of yet-unidentified factors binding to the methylated p53 and interfering with Mdm2-dependent ubiquitination (Chuikov *et al*. 2004).

This hypothesis was supported by the findings of Ivanov *et al*. (2007) in which stabilization of p53 and promotion of its transcription factor activity was seen to occur after methylation by Set7/9, with no effect on p53's DNA-binding activity observable. To test if this methylation event induced stabilisation via direct elimination of subsequent ubiquitination (a modification known to label p53 for degradation), wild-type and K372R (lysine-to-arginine) mutant ectopic p53 were tested for relative efficiencies of *in vivo* ubiquitination. However, similar ubiquitination levels were observed, nullifying this hypothesis. Comparison with histone biology revealed an alternate, indirect mechanism for interference with ubiquitination. Similar to methylation of K4 residues increasing K9 and K14 acetylation in histone H3, it was demonstrated that lysine methylation of p53 is imperative for downstream acetylation at proximal C-terminal lysine residues (Zegerman *et al*. 2002, Ivanov *et al*., 2007). While use of small hairpin RNA (shRNA) to silence Set7/9 expression was found to have no effect on histone H3 methylation, methylation of p53K372 was diminished, along with DNA damage-induced acetylation of p53. Thus, methylation of p53K372 stimulates acetylation, providing a mechanism for the obstruction of ubiquitination. This methylation-acetylation interplay not only stabilises and activates p53 by blocking its ubiquitination, it also promotes acetylation of histone H4 at, at least, the promoter of the canonical p53 target gene, *p21*. Jointly, these events up-regulate *p21*, ultimately leading to cell cycle arrest.

Complexity of Methylation-mediated Regulation Suggests a 'p53 Code'

Aside from this complex interplay with other modifications, Set7/9-mediated methylation of p53K372 has also been shown to

be an activating event, due to its inhibitory role in the prevention of Smyd2-mediated methylation of lysine 370, a monomethylation event with a repressive effect on p53 activity (Huang *et al*., 2006). This web of interacting modifications is suggestive of a 'p53 code', as no one event is acting as an independent on/off switch for p53 activity.

As well as establishing that monomethylation of K370 (K370me1) represses p53 function, this collaboration also revealed that dimethylation of K370 (K370me2) activates p53 function (Huang *et al*., 2007). Although the distinct methyltransferase responsible for this event was not identified by their work, discovery of the first incidence of demethylation of a non-histone protein was shown in this study, with demonstration that the histone lysine-specific demethylase LSD1 demethylates p53 at K370. While *in vitro* study showed demethylation of K370me1 and K370me2, LSD1 was shown to have a strong preference for demethylation of K370me2 *in vivo.* According to knowledge of the consequences of different lysine methylation states in histones, effector proteins of the K370 methylation states were investigated. Using a GST protein domain microarray, the tandem Tudor domains of the p53 co-activator p53-binding protein 1 (53BP1) were found to bind preferentially to K370me2 (compared with K370me0, K370me1 or K370me3). Strong evidence of LSD1-mediated modification of p53K370me2, and not p53K370me1, resulting in regulation of p53 activity via obstruction of p53-53BP1 interaction, was supplied via direct assay experiments, adding to both the known complexity of methylation-mediated regulation of p53 and to the idea of post-translational modifications acting as a fine-tuner for p53 activity (Huang *et al*., 2007).

Lysine methylation of p53K382 & the Drawbacks of Current Experimental Techniques

SET8/PR-Set7 (Set8) is a histone methyltransferase that has been established to monomethylate histone H4 tails at K20 (Fang *et al*. 2002, Nishioka *et al*. 2002b). SET8-mediated regulation of *p21* and *PUMA*, in response to DNA damage, has been determined to be due to direct activity as a p53 methyltransferase, and not due to

H4K20 methylation effects. Lysine residue K382 was identified as SET8's target methylation site, and confirmed *in vitro* via ablation of SET8-mediated methylation, solely upon substitution of K382, and *in vivo* via decreases in p53K382me1 after SET8 knockdown by RNA interference (RNAi). p53K382me1 levels decrease following DNA damage, corresponding with functional characterization as a mark that contributes to the inhibition of p53-mediated transcriptional activation (Shi *et al*. 2007). MBT domain-containing proteins have been demonstrated to show preferential binding for monomethylysines on histone proteins (Kim *et al*. 2006). As lysine methylation alters the surface architecture of p53, and thus should achieve this regulatory activity via influence on interaction with a distinct protein binding partner, interaction with a Malignant Brain Tumor (MBT) domain-containing protein, was hypothesised to explain the molecular mechanism by which p53K382me1 acts to repress p53 (Shi *et al*. 2007).

L3MBTL1 is an established member of the polycomb group proteins. As a transcriptional repressor it promotes compaction of chromatin, enhancing inaccessibility of areas enriched for mono- and dimethylated histone marks (Kalakonda *et al*. 2008). Sequence similarity between the L3MBTL1-target H4K20 and p53K382 led to subsequent identification of L3MBTL1 as this MBT-containing protein. Addition of a single methyl moiety at K382 promotes p53-L3MBTL1 interaction, resulting in inhibition of p53 transcriptional activation by subsequent stabilization of L3MBTL1 occupancy at the *p21* (and *PUMA)* promoter. This phenomenon was shown in the absence of DNA damage, to ensure no effects from alternate mechanisms of *p21* regulation were recorded. Upon depletion of L3MBTL1 by RNAi, up-regulation of *p21* transcript levels was found in p53-containing cells, but not in the absence of p53. Thus, p53 target gene repression was suggested to be achieved due to p53-mediated stabilisation of L3MBTL1 at chromatin locations of these genes. Accordingly, upon DNA damage, reduction in p53K382me1 levels abolishes the p53-L3MBTL1 interaction, resulting in disassociation of L3MBTL1 from the p53-high response targets, allowing immediate activation by the adjacent, hitherto quiescent p53 (West *et al*. 2010).

In contrast to L3MBTL1 Flag-immunoprecipitates (IPs), it was noted that the P53-L3MBTL1 interaction was not affected by DNA damage in whole cell extracts prepared from 293T cells. 293T cells contain the simian virus 40 (SV40) larger T antigen, which stabilises and inactivates p53, resulting in high levels of endogenous p53 (Fu *et al*. 2010). It is unclear whether p53 levels are unaffected by DNA damage in these cells due to this altered p53 activity or whether unknown interactants in the whole cell extract dampen this DNA damage response. To avoid such issues, p53 activity should, ideally, be investigated in cell lines in which p53 activity is not already altered. Furthermore, the decrease in occupancy of L3MBTL1 at the *p21* promoter is ~15-20 lower than the increase in p53 occupancy. The reason for this discrepancy is not discussed and presents an area of further study, in which determination of the mechanism behind this amplification of p53 occupancy may shed further light on the p53-L3MBTL1 relationship.

Contrary to the functional characterisation of SET8 as a negative regulator of p53 activity, SET8-dependent p53-K382me1 binding on the promoter of GADD45 was found to be up-regulated in response to DNA damage. GADD45's role in DNA repair thus indicates that methylation of K382 may play a more complex role than a simple on/off switch. Further studies are needed to explore its proposed role as a mechanism for predisposing p53 to mild cellular stresses, allowing a shift in p53-mediated transcription towards DNA repair without initiation of apoptosis (Shi *et al*. 2007).

Demethylation of p53: A Knowledge Void

Apart from LSD1, no additional p53 demethylases have been conclusively defined. Reduction of p53K372me1 levels have been recorded during p53 activation, suggesting this mark is subject to demethylation (Huang & Berger 2008). While the histone lysine demethylase JMJD3/KdM6b has been suggested to be responsible for this demethylation event, direct evidence of p53 demethylation by JMJD3 was not shown in this study (Ene *et al*. 2012). Furthermore, Williams *et al*. (2014) were unable to show demethylation of p53 peptides by JMJD3 *in vitro*, suggesting JMJD3 does not act as a p53

demethylase, but may instead act on p53 responsive elements, via interaction with p53. Alternatively, demethylation of currently unidentified p53 methylation sites by JMJD3 cannot be ruled out by either of these studies. As shown by Cao *et al*. (2013), new methods of *in vivo* large-scale surveys may yet identify many more lysine methylation sites in p53, making the likelihood of an unknown JMJD3 demethylation site in p53 much more probable.

JMJD2c and PLU-1, like JMJD3, are members of the JmjC domain-containing protein family (He *et al*. 2012). Both proteins have been characterised as histone demethylase, with JMJD2c responsible for H3K9me3/me2 demethylation, and PLU-1 responsible for demethylation of H3K4me (Cloos *et al*. 2006, Yamane *et al*. 2007). Changes in the expression of JMJD2c and PLU-1 have both been found to have no substantial effect on p53 levels, indicating that these demethylases are not likely to demethylate p53, even through currently unidentified methylation sites (Yamane *et al*. 2007, Ishimura *et al*. 2009). Therefore, expansion of current knowledge surrounding p53 demethylation must look beyond these histone demethylases to untested demethylases or to completely novel regulators of p53.

Conclusions

It is clear that post-translational modifications regulate p53 and histones via the same set of underlying principles. Just as methylation regulates the activity of histone proteins via alteration of their ability to interact with a range of effector proteins, so too does methylation regulate the activity of p53 via modulation of its capacity to associate with a range of interaction partners. These modification processes are also highly interconnected, lysine methylation 'writer' proteins, including Set7/Set9, Set8, and LSD1 show functionally overlapping activity, as do the 'reader'/effector proteins L3MBTL1 and 53BP1. Thus, we can see that apparent parallels allow elucidation of the function of unannotated modifications in p53, by drawing comparison to partner histone modifications. While this technique may not yet have resulted in successful characterisation of additional p53 demethylase proteins, examination of untested histone demethylases presents an area where future investigation

is likely to yield some significant results. It is also important to note that the discovery of novel p53 methylases or demethylases could uncovered unidentified histone-targeting proteins.

The limitations of experimental techniques can beget inconsistent or inconclusive, results as evident in the characterisation of p53-L3MBTL1 interaction. Moreover, investigation of p53 *in vitro* can lead to incorrect characterisation of modifications or their interactants. Transfection stress, aberrant ratios of p53 and its regulators or targets, and forcing of nonphsiologic interaction can result in the deviant phenotypes from which these characterisations arise. These phenomenon can occur due to overexpression of one or more proteins within the system, due to the selective pressure of cell culture environments, or simply from the use of cancer cell lines, such as the 293T cell line (Toledo & Wahl 2006, Love & Grossman 2012). Validation of L3MBTL1's association with p53, and of any putative modification, by investigation of activity in *in vivo* mouse models or physiologically relevant cell culture environments, must become an essential component of experimental procedure in order to best circumvent these shortcomings. As assay development and mouse models become more sophisticated, including the development of human p53 knock-in 'HUPKI' mouse models, this requirement will become less of a challenge, as even physiological distinctions between humans and mice can be overcome (Luo *et al*. 2001).

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