REGULATION OF P53 FUNCTION BY LYSINE METHYLATION

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Abstract

The human p53 tumor suppressor is the most widely studied gene and protein in cellular biology. An open question in the field relates to the regulatory mechanism(s) that manage the complex functions of an essential tumor suppressor protein. Recently, a number of the protein lysine methyltrasferases (PKMTs) that mediate histone lysine methylation have also been identified as having activity on non-histone substrates. Reversible covalent methylation of lysine residues on histone or non-histone proteins constitutes a principal molecular mechanism that guides substrate proteins to diverse biological outcomes. The goal of this work was to 1) expand the understanding of lysine methylation and methyllysine recognition in the context of non-histone tumor suppressor proteins, and 2) elucidate the molecular mechanisms of such events.

A handful of studies characterizing lysine methylation events on four distinct lysines in the C-terminal regulatory region of p53, have lead to the emergence of p53 as a model for non-histone lysine methylation. We contributed to this body of work by demonstrating that the lysine methyltransferase enzyme SET8/PR-Set7 is a novel regulator of p53. SET8 monomethylates p53 at lysine 382 (p53K382me1), resulting in reduced transactivation of highly responsive target genes. We used biochemical and crystallographic studies to identify the triple malignant brain tumor (MBT) domain containing chromatin compaction factor L3MBTL1 as the second know p53-effector protein. We demonstrated that SET8-mediated methylation of p53 at K382 promotes the interaction between L3MBTL1 and p53 in cells, and the chromatin occupancy of L3MBTL1 at p53 target promoters, thus providing a molecular explanation for the mechanism by which p53K382me1 is transduced to regulate p53 activity.

Additionally we demonstrated that retinoblastomal tumor suppressor (RB) can be mono-methylated in vivo by SMYD2 at lysine 860, and that this methylation provides a direct binding site for L3MBTL1, helping to guide its functions in mammalian cells.

In summary, we identified SET8 as a novel p53-modifying enzyme that mediates the regulatory post-translational modification at lysine 382; and, we
identified L3MBTL1 a novel methyllysine effector protein for that mark. In a collaborative study we demonstrated that methylation of RB by Smyd2 promotes interaction with L3MBTL1. Together these results suggest that regulation by lysine methylation represents a broader paradigm for modulation of non-histone protein activity.
Preface

This thesis is a compilation of collaborative work, and people who contributed are listed at the beginning of each chapter. My specific contributions to each chapter are as follows:

Chapter two: I treated multiple cell lines with various DNA damage reagents to demonstrate that SET8 mRNA and protein levels are reduced upon DNA damage, while levels of a second methyltransferase for p53, Smyd2, are not.

Chapter three: I characterized the in vitro and in vivo binding between p53K382me1 with peptide pulldown assays, and cotransfection and co-immunoprecipitation respectively. I used chromatin immunoprecipitation of flag-L3MBTL1 and endogenous p53 to establish the presence of these proteins at two p53 target genes. I performed L3MBTL1 knockdowns with siRNA in two different cell lines to demonstrate that the release of p21 from repression is dependent on the presence of p53. I generated U2OS cells stably expressing L3MBTL1 shRNA and made growth curves and performed colony formation assays to show that L3MBTL1 depletion attenuates cellular proliferation rates. Lastly, I performed endogenous co-immunoprecipitation of L3MBTL1 to show that DNA damage treatment reduces the in vivo association of p53 and L3MBTL1; and, that reduction of SET8 by siRNA produces a similar effect.

Chapter four: I made the flag-L3MBTL1 mammalian expression vector and GST-3XMBT wild type and mutant constructs used in this study.

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Chapter 1: Introduction
Regulation of p53 function by lysine methylation

The human p53 tumor suppressor is the most widely studied gene and protein in all of molecular biology. By no small coincidence it is also the gene most frequently mutated in human cancers, at a rate of 50% by most estimates (1). As the much-heralded “guardian of the genome” p53 is subjected to intense scientific scrutiny. Its many jobs entail a momentous responsibility for one protein, a point superbly captured in a humorous anecdote detailing the psychological affairs of p53. To his psychologist, “Every little thing I do is watched, every decision I make is monitored…I have regulations, modifications, physiological partners that rely on me!” (Tsvetkov and Dekel, http://www.lablit.com/article/621). It is a lot of pressure on one protein; indeed, such an important factor is meticulously managed in the cell.

One well established mechanism of p53 regulation takes place at the protein level. Under basal conditions, p53 is rapidly degraded by the ubiquitin ligase mdm2. Genome instability and other diverse cellular stresses lead to p53 activation through a host of mediators such as ATM, CHK2, and p19ARF (2-4). Various effector proteins then stabilize p53 to support downstream activity including activation of distinct gene expression programs that result in DNA repair, cell cycle arrest, or programmed cell death. Ultimately p53 guards cells not only against malignant progression but also plays a role in developmental processes as diverse as aging, differentiation, and fertility; and numerous pathologies including neurodegenerative disorders, diabetes, and myocardial infarction, to name just a few (5-7). The myriad processes in which p53 is involved highlight a pervasive question pertaining to the regulation and specification of p53 activity. How is one protein with so many essential functions directed to the right places at the right time? What regulatory mechanism(s) manage the staggering complexity of a single tumor suppressor protein?

To begin to answer these questions we look to the traditionally epigenetic mechanism, post-translational modification (PTM), for the complex combinatorial potential that it imparts upon protein substrates. p53 is decorated with numerous PTMs including phosphorylation, acetylation, ubiquitination and more; but, of
particular interest in this work is lysine methylation as a regulatory modification. Historically, lysine methylation has been most well studied on histone proteins. In humans, a group of SET domain containing protein lysine methyl transferases (PKMTs) target lysines in the unstructured N-terminal histone tails inscribing mono-, di-, or trimethyl marks. The different lysine methyl marks are reversed by a second class of demethylase enzymes. Switching between methylation states represents a dynamic process that results in changes in chromatin structure and gene expression (8,9). Lysine methylation changes neither the conformation, nor overall charge of substrate proteins; rather it is increasingly understood to modulate the surface architecture of histone tails leaving a mark that establishes an interaction template for the binding of effector protein(s). For instance, H3K4 dimethylation (H3K4me2) is recognized by WDR5 in the context of an MLL family complex; this interaction enables further methylation by MLL-family PKMT complexes (10). Another methyl state may attract an entirely different set of effectors: H3K4 trimethylation (H3K4me3) serves as a template for recruitment of histone deacetylase complexes. During DNA damage, recognition of H3K4me3 by ING2 results in recruitment of Sin3/HDAC and repression of cell proliferation genes (11). H3K4me3 is also recognized by the PHD finger of histone demethylase PHF2, resulting in PHF2-dependent promoter demethylation and expression of ribosomal genes (12). This illustrates the point that interaction of a single mark with different effector proteins leads to divergent functional outcomes, and that specific conditions determine the timing and identity of the interaction partner. These examples also highlight the occurrence and complexity of cross talk between post translational modifications on histones.

Recently, a number of the PKMTs that mediate histone lysine methylation have also been identified as having activity on non-histone substrates (8,9,13). A handful of studies characterizing lysine methylation events on four distinct lysines in the C-terminal regulatory region of p53, have lead to the emergence of p53 as a model for non-histone lysine methylation. Similar to the N-terminal tails of histones, the p53 C-terminal tail is an unstructured region containing a number of lysines available for
PTM (Fig. 1-1). Furthermore, as discussed in this thesis, the aforementioned mechanisms regulating histone biology are currently being extended to p53, thus demonstrating that fine-tuned regulation through PTM is an attractive explanation for ability of p53 to participate in innumerable cellular functions. In essence, lysine methylation allows p53 to be in more than one place a time. It is useful to think of p53 not as a uniformly consistent entity, but rather as a dynamically regulated population with multiple contingents spontaneously created by PKMT’s in response to various stimuli and subsequently diverted to various downstream functions by a host of effector proteins.

The known occurrences and mechanisms by which lysine methylation regulates p53 activity are discussed in detail below. Parallels between a PKMT’s activity on p53 and its activity on histones are also considered.
Figure 1-1 – Summary of the known lysine methylation events on p53

Schematic of p53 showing sequence of C-terminal unstructured regulatory domain. Protein lysine methyltransferases are shown in blue; [brackets] indicate concentration of various methylated p53 populations. Effector proteins shown in yellow. Red and green backgrounds represent p53 off and on states respectively.

SET7/9 monomethylation at lysine 372 stabilizes p53

Set7/9 is a SET domain containing methyltransferase with monomethylation activity first characterized on H3K4, a mark associated with gene activation (14,15). Set7/9 mediated H3K4me1 disrupts the association of histones with the histone deacetylase repressor complex NuRD (15,16). H3K4me1 also inhibits the methylation of H3K9 by Suv39H1, a known chromatin repressive modification (15,17). Set7/9 recognizes a
conserved K/R-S/T/A motif, though the enzyme’s sequence specificity does not appear to be strict since the target lysine in some substrates, such as PCAF and RelA, are not found in the motif (18). To date, Set7/9 has the most non-histone targets of any human methyltransferase. Reviews that discuss Set7/9 methylation of diverse non-histone targets have recently been published (18,19). In general, and parallel to Set7/9 activity on its histone substrate, themes of transcriptional activation and stability have emerged. Indeed, these themes are echoed in Set7/9 activity on p53, as described below.

In 2004 Chuikov et al cracked open the field of non-histone protein lysine methylation with the identification of p53 as a substrate of SET9 monomethylation (20). Very few examples of PKMT activity on non-histone substrates existed previously (21,22), and this work paved the road towards identification of other non-histone substrates, not only of Set9 but also for other PKMTs with activity on diverse substrates. The SET9 methylation site on p53 was mapped \textit{in vitro} using p53 truncations and KtoR mutants of p53 C-terminal lysines, and confirmed \textit{in vivo} by a p53K372me1 specific antibody. It was shown that upon DNA damage, SET7/9 methylates p53K372 in the nucleus resulting in global stabilization of chromatin bound p53. Increased promoter occupancy and transcriptional activation of p21 and other target genes were observed (20).

Follow up studies were able to attribute the Set7/9 methylation mediated increase in p53 protein stability, transactivation activity at p21, and G2/M cell cycle arrest to subsequent acetylation at C-terminal lysine residues (23). Side effects of Set7/9 methylation of H3K4 were ruled out since 1) Set7/9 IP’d from cells treated with adriamycin was able to methylate p53 but not nucleosomes and 2) H3K4me1 levels upon adriamycin treatment were unaffected by Set7/9 knockdown (23). Interestingly these experiments illuminated the fact that DNA damage potentiates Set7/9 activity specifically on p53, but not on it’s histone target H3K4. Analysis of mRNA, protein, and ChIP samples following DNA damage induced by adriamycin elucidated a dynamic sequential relationship between p53K372 methylation and p53 acetylation, with the former preceding the latter (23). These experiments
demonstrated the following scenario: DNA damage increases Set7/9 mediated p53K372mel levels at the p21 promoter. The methylation subsequently promotes acetylation by p300, which prefers methylated, DNA bound, tetrameric p53 vs. unmodified, unbound p53 as a substrate. Localized p300 also methylates histone target H4 leading to a concerted gene activation state mediated by activated acetylated p53 and acetylated opened chromatin (23). Set7/9 thus coordinates gene activation through concerted action on chromatin bound p53 and indirect activating acetylation of chromatin.

The creation of a Set7/9 null mouse lead to further insight into the methylation–acetylation interplay propagated by Set7/9 methylation of p53 in vivo. Set7/9 knockout mice fail to methylate p53 at K369, the equivalent of K372 in human p53 (24). It was found that the in vivo association of the Tip60 acetyltransferase is dependent upon K369 methylation, and no p53 acetylation was detected in bone marrow of Set7/9 null mice following DNA damage. Cells from the Set7/9 knockout mice are unable to induce p53 downstream targets (p21) upon DNA damage, and deletion MEFs are more susceptible to transformation upon addition of ras oncogenes compared with wildtype (24). Thus, Set7/9 behaves like a tumor suppressor by positively regulating p53 activity.

The precise mechanistic details of p53 stabilization through Set7/9 methylation mediated enhancement of acetylation can still only be speculated upon thus far. Lysine acetylation in general counteracts ubiquitination at the same residues in the C-terminus. Promotion of acetylation by K372 methylation at other C-terminal p53 lysines may thus block ubiquitination and subsequent degradation (23). The discovery of a second methylation event mediated by Smyd2 at p53K370 shed light on another possible role for Set7/9 mediated stabilization of p53: inhibition of a negative methylation mark in order to activate p53 upon DNA damage (25).

**Repression of p53 activity by Smyd2-mediated methylation**

In contrast to the activating effect of SET7/9 methylation at K372, the next methylation event documented on p53, Smyd2 mediated mono-methylation at lysine 370, was shown to have a repressive effect on p53 transactivational activity (25).
repressive role for Smyd2 in p53-mediated cell cycle arrest and apoptosis was defined owing to several key observations made in the Huang et al study: (1) the methylation event was shown to effectively reduce p53 occupancy at two p53 target gene promoters: p21 and mdm2, and (2) elimination of Smyd2 following different types of DNA damage treatment ramped up p53 mediated apoptosis.

Numerous examples of cross-talk between histone modifications and the obvious proximity of lysines 370 and 372 indicated a potential for cross-talk taking place between these methyl marks at these lysines. Indeed, both in vitro and in vivo data suggest that SET7/9 mediated methylation of K372 inhibits Smyd2 mediated methylation of K370 (25). Cotransfection of SET9 together with flag-p53 and Smyd2 in H1299 cells decreases the p53 and Smyd2 interaction observed by flag immunoprecipitation suggesting that SET9 physically blocks Smyd2 binding to p53 (25). Thus at least part of the positive effect of SET7/9 methylation on K372 may be explained through its inhibitory role in preventing Smyd2 and p53 interaction.

Interestingly, Smyd2 monomethylation activity on p53 under normal conditions and SET7/9 activity on K372 potentiated upon DNA damage appear to regulate an equilibrium between free p53 and promoter bound p53 species (25). A Smyd2 generated pool of p53K370me1 has reduced promoter occupancy thus resulting in low p53 promoter concentrations under normal conditions. Upon DNA damage, activation of SET7/9 on p53 increases concentrations of p53K372me1 which inhibit the Smyd2 – p53 interaction. p53K372me1 promotes subsequent acetylation, increases stability through competitive inhibition of ubiquitination, and leads to increased promoter occupancy and transactivation of target genes (25).

**p53 is regulated by the lysine demethylase, LSD1**

The story for regulation of p53 through Smyd2 methylation at K370 does not stop at the level of monomethylation. p53 can also be dimethylated at K370 by an as yet unidentified methyltransferase (26). Dimethylation was shown to have a positive effect on p53 since the K370me2 species increases at target promoters upon DNA damage (26). The positive role of K370 dimethylation is thought to be regulated through binding between p53K370me2 and 53BP1 via the tudor domain. That 53BP1
was identified as an effector protein for p53K370me2 is not too surprising given its previously characterized role as a transcriptional coactivator of p53 (27) and as an effector of dimethylated histone marks including H3K79 and H4K20 (28,29).

Demethylation from the activating dimethyl to the inhibitory monomethyl state is regulated by the lysine specific demethylase LSD1 (26). This was a novel finding since demethylation had not been previously observed on non-histone proteins. During DNA damage LSD1 depletion significantly increased only the K370me2 signal, suggesting that LSD1 primarily removes the dimethyl mark in vivo (26). Consistent with its role in maintaining a repressive chromatin environment through histone demethylation activity (30,31), LSD1 represses p53 function by limiting the accumulation of p53K370me2 through dynamic demethylation thus converting the active dimethyl species to the inactive monomethyl species (26). p53K370me1 is unable to interact with coactivator 53BP1 and exhibits a reduced capacity for association with target gene promoters. Under conditions requiring gene activation, an unidentified methyltransferase increases the population of p53K370me2 thereby supporting a stabilizing interaction with 53BP1 and leading to transcriptional activation of target genes (26).

**SET8 monomethylation at lysine 382 & interaction with L3MBTL1**

In 2007 a third physiologically relevant methylation event on p53 was described: monomethylation of lysine 382 by SET8 (32). SET8 (PR-SET7/KMT5a) is the PKMT for H4K20me1, a mark which is, not without exception (33,34), generally understood to promote gene silencing and condensed chromatin states in mammals (35-39). Roles for SET8 in such diverse cellular processes as S-phase progression (37,40), mitosis (41,42), and DNA-damage checkpoint signaling (43,44) have been described. Interestingly the themes of transcriptional repression and DNA-damage checkpoint signaling are shared in SET8 regulation of p53.

The SET8 mediated monomethylation of p53 at lysine 382 represents the second example of a repressive mark that affects p53-mediated transcriptional regulation. SET8 methylation on p53 was predicted, in part, by consensus in the amino acid sequence at the substrate site of SET8 in H4 (K20) and the residues surrounding lysine
382 in the p53 C-terminus (32). The restraining effect of SET8 on p53 transcriptional activation of p53 highly response targets p21 and PUMA was teased out through SET8 knockdown and overexpression. Decreasing SET8 levels increases p53 occupancy and transcriptional activity at target genes, while overexpressing SET8 induces the opposite state, decreased occupancy and reduced transactivation mediated through wild type p53 (32). Since SET8 is present under normal conditions but repressed upon DNA damage it thus serves as a modulator of p53 activity, generating an inert population, monomethylated at K382, that suppresses p53 transactivation activity at highly responsive targets in the absence of DNA damage. SET8 thus promotes survival of healthy cells through methylation-mediated repression of its non-histone target p53. These phenotypes were shown to be a consequence of SET8 methylation of p53, and not an indirect effect of H4K20 methylation by SET8 since H4K20me1 levels were not changed, and expression effects on specific target genes were abolished upon p53 knockdown (32).

SET8 monomethylation at lysine 382 represents another example of how lysine methylation prepares a substrate for interaction with an effector protein. While it was observed that SET8 negatively regulates acetylation at the K382 residue – SET8 overexpression in U2OS cells reduced the amount of acetylation detected by p53K382Ac antibody upon NCS treatment – this was not thought to be the chief mechanism by which methylation represses p53 transactivational activity, since a p53K382R mutant activates target genes with an efficacy comparable to the wild type protein (32). Interestingly, sequence similarity between the H4 tail at K20 and p53 at K382 was predicative not only of the PKMT found to methylate these residues, but also of two interacting effector proteins subsequently identified: L3MBTL1 and 53BP1.

The malignant-brain Tumor (MBT) protein L3MBTL1 was recently identified as an interaction partner for p53K382me1 (45). L3MBTL1 binds H4K20me1 (and other monomethylated histones, refs) via its middle MBT repeat and compacts chromatin in a histone lysine methylation dependent manner (35,46). Recent work shows novel methylation dependent interactions between L3MBTL1 and non-histone
targets p53 and RB by virtue of the same MBT2 mediated binding modality observed in interactions with methylated histones (45,47). In fact, p53 joins a growing list of non-histone interaction partners for L3MBTL1 including TEL and RB, each of which utilizes L3MBTL1 for its transcriptional repressing capability at respective target promoters (35,48).

The p53 – L3MBTL1 interaction is driven in vivo by SET8 mediated methylation of p53 at lysine 382 (45). Under normal conditions, p53K382me1 is thought to stabilize L3MBTL1 occupancy at the p21 promoter. In some cases, L3MBTL1 mediated repression of target genes appears not to need transcription factor guidance to chromatin. For example, PR-SET7 mediated monomethylation of H4K20me1 in the cyclin E1 and RUNX1 promoters is sufficient for L3MBTL1 mediated transcriptional repression of these genes (36,38). However, in the case of the highly responsive target p21, L3MBTL1 promoter occupancy was observed to have some dependence on the presence of p53; L3MBTL1 occupancy at the p21 promoter was not observed in a p53 null cell line (45). DNA damage induced by NCS treatment leads to repression of SET8 levels, reduction of p53K382me1, dissociation of p53 from L3MBTL1, and reduced L3MBTL1 occupancy at the p21 promoter (45). p53K382me1 interaction with L3MBTL1 thus enables quiescent p53 occupation at promoters of highly responsive target genes where it sits poised for immediate activation upon DNA damage.

53BP1 interaction with p53K382me2

Kachirskia et al found that the tandem tudor domains of 53BP1 recognize and bind to a second dimethylated p53 residue, p53K382me2 (49). Incidentally, 53BP1 is the second effector protein (along with L3MBTL1) shared between K382 of p53 and K20 of histone H4. The tandem tudor domains of 53BP1 bind to histone target H4K20me2 allowing it to accumulate at double strand breaks (DSB) (29). p53K382me2, a species that increases with DNA damage, is similarly recognized by the tandem tudor domains of 53BP1 (49). This interaction stabilizes p53 by positively regulating the accumulation of p53 protein that accompanies DSB lesion formation. The enzyme responsible for p53K382 dimethylation is as of yet unknown. In light of the small
collection of interacting proteins shared between these substrates, one may speculate that the enzymes responsible for H4K20me2, Suv4-20h1/h2 (50), might also include p53 as a substrate. However, a screen of more than 30 lysine methyltransferases for dimethylation activity on p53K382 did not identify Suv4-20/h1/h2 or any other PKMT candidates as writers of this modification (49). The identity of PKMTs that mediate K370me2 (discussed above) and dimethylation of the novel species of unidentified function, K386me2, are also forthcoming.

**G9a and Glp methylate p53 at lysine 373**

The related lysine methyltransferases G9a and GLP form a stoichiometric heteromeric complex that mediates H3K9 dimethylation *in vivo* with each capable of independent H3K9 methylation *in vitro* (51,52). The most recent work on lysine methylation of p53 has identified G9a and Glp as mediators of dimethylation of p53 lysine 373 (53). A p53K373R mutant cannot be methylated, suggesting that lysine 373 is the only site methylated by G9a/Glp. In contrast to the activating effect of dimethylation at lysines 370 and 382, mediated through interaction with 53BP1 as described above, p53K373me2 represents an inactivating dimethyl mark on p53. This was concluded based on the observation that 1) p53K373me2 levels do not increase in response to at least one type of DNA damage treatment (adriamycin), and 2) transient knockdown of either G9a or Glp by siRNA resulted in p53 activation as measured by an increase in the proportion of cells undergoing apoptosis (53). Since combined knockdown of G9a and Glp does not further increase the percentage of apoptotic cells it may be inferred that G9a and Glp act synergistically to repress p53 through dimethylation at lysine 373.

The molecular mechanism of repression for K373me2 has not yet been characterized. In the case of histones, G9a/Glp mediated mono and dimethylation of H3K9 mediate transcriptional repression in part through recruitment of HP1 protein to chromatin (51).
References


Chapter Two: Modulation of p53 function by SET8-mediated methylation at lysine 382

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Summary

Reversible covalent methylation of lysine residues on histone proteins constitutes a principal molecular mechanism that links chromatin states to diverse biological outcomes. Recently, lysine methylation has been observed on non-histone proteins, suggesting broad cellular roles for the enzymes generating and removing methyl moieties. Here we report that the lysine methyltransferase enzyme SET8/PR-Set7 is a novel regulator of the tumor suppressor protein p53. We find that SET8 specifically monomethylates p53 at lysine 382 (p53K382me1). This methylation event robustly suppresses p53-mediated transcription activation of highly responsive target genes, but has little influence on weak targets. Further, depletion of SET8 augments the pro-apoptotic and checkpoint activation functions of p53, and accordingly, SET8 expression is downregulated upon DNA damage. Together, our study identifies a novel p53-modifying enzyme, a new regulatory post-translational modification of p53, and begins to dissect how methylation may contribute to a dynamic post-translational code that modulates distinct p53 functions.
**Introduction**

Methylation events at distinct lysine residues within histone proteins are linked to diverse functional outcomes (1). For example, methylation at histone H3 at lysine 4 (H3K4me) is largely detected at euchromatin and is thought to generally lead to increased DNA accessibility, whereas methylation of histone H3 at lysine 9 (H3K9me) is most commonly associated with heterochromatin and inaccessible DNA (2). One mechanism by which lysine methylation aids in the establishment of distinct chromatin states is by mediating modular protein-protein interactions (3). In this regard, the proteins that recognize a methylated lysine within a specific sequence context can define the functional outcome of a lysine methylation event. Further, histone lysines can be mono-, di- or trimethylated, with a unique activity frequently being coupled to the specific state and extent of methylation on the lysine residue. Thus, methylation of lysine residues on a target protein can increase the signaling potential of the modified protein and as such lead to diverse physiologic consequences.

p53 is a transcription regulator that plays a central role in tumor suppression by directing cellular responses to diverse stresses (4,5). The levels and activity of p53 are regulated by a complex network of post-translational modifications that primarily occur within two regions of the protein: an N-terminal region that is phosphorylated at multiple sites and a C-terminal region rich in basic residues (5,6). Recent reports indicate that p53 is monomethylated at two different lysine residues within the regulatory C-terminal region (7,8). Akin to how H3K4me and H3K9me are linked to opposing states of chromatin, the two known sites of p53 methylation are coupled to activities that oppose one another. Specifically, SET7/9-mediated monomethylation of p53 at K372 (p53K372me1) activates p53, postulated in part to occur via stabilization of chromatin-associated p53, whereas Smyd2-mediated monomethylation of p53 at K370 (p53K370me1) represents a repressive mark, the generation of which is impeded by p53K372me1 (7,8). In addition to methylation at K370 and K372, the C-terminal region of human p53 harbors several K residues that are subject to modification by acetylation, ubiquitylation, sumoylation and neddylation (reviewed in Toledo and
Wahl, 2006 (5). Notably, endogenous p53 protein from two independent mouse models in which these lysines were targeted for mutation did not display an alteration in stability, and the phenotypes of cells derived from the mice were relatively mild (9,10). This work argues that in sum, the post-translational modifications (PTMs) on the p53 C-terminal region fine-tune p53 activity. However, as substitution of lysines will prevent all forms of PTMs, including mono-, di- and trimethylation, mutant phenotypes may indicate the elimination of both positive and negative regulatory effects. Thus, identifying and characterizing the enzymes that catalyze p53 modifications is critical for developing a molecular understanding of how p53 PTMs are coordinated to regulate p53 functions.

SET7/9 and Smyd2 were both first reported to function as histone methyltransferases (HMTs), suggesting that other HMTs might have non-histone substrates (11-13). SET8/PR-Set7 is an HMT that adds a single methyl moiety to histone H4 tails at lysine 20 (H4K20me1), preferentially to nucleosomal H4 (13,14). Mutation of the SET8/PR-Set7 gene in Drosophila melanogaster leads to lethality during development (13). H4K20me1 generation by SET8 has also been shown to be important for gene silencing and mitotic regulation (14-16). Here we demonstrate a novel activity for SET8 as a p53 methyltransferase. We find that SET8-mediated methylation of p53 at K382 represses highly responsive p53 target genes to attenuate p53 pro-apoptotic and cell-cycle arrest functions. We propose a model in which SET8-mediated p53 methylation tips the balance of p53 function away from cell elimination towards cell survival.

Results

*In vitro identification of SET8 as a p53K382 monomethyltransferase.* To screen known HMTs to determine if they might function as p53 methyltransferases, we expressed recombinant SET7/9, Suv39h1, hDOT1L, SET8/PR-Set7 and Suv4-20h1, and performed *in vitro* methylation assays using full-length GST-p53 and histones as substrates (Fig. 2-1A). As expected, SET7 methylated p53 and histone H3, but not nucleosomes(Fig. 2-1A) (7). The other enzymes showed methyltransferase activity on
their cognate histone substrate, but only SET8 was found to possess methylation activity on p53 (Fig. 2-1A). We performed ms/ms analysis of the methylated p53 protein and mapped K382 as a residue modified by SET8 (data not shown). To confirm these results, we generated several single site mutants in the context of full-length p53, and performed in vitro methylation assays with SET8 and SET7. As shown in (Fig. 2-1B), substitution of K382 with arginine (p53K382R) abolishes SET8-mediated p53 methylation, but has no effect on SET7-mediated activity. Substitutions at other lysines of p53 had no impact on SET8 methylation (Fig. 2-1B). Based on these data, we conclude that SET8 methylates p53 exclusively at lysine 382.
Figure 2-1 – SET8 monomethylates p53 at K382 in vitro

(A) Identification of SET8 as a novel p53 methyltransferase. Autoradiograms of methyltransferase assays with the indicated recombinant HMTs and substrates. (B)
SET8 methylates p53 at K382. Autoradiograms of methyltransferase assays with SET8 or SET7 on wild-type p53 or the indicated p53 mutants. (C) Alignment of amino acid sequences of SET8 substrates histone H4 (aa 1–24) and p53 (aa 367–389). Asterisk indicates SET8 methylation sites on H4 and p53. (D) p53 is monomethylated by SET8 at K382. Mass spectrometry analysis of p53 peptide (aa 367–389) before (left panel) and after (right panel) SET8 methyltransferase reaction.

SET8 is a strict monomethyltransferase for H4K20 (17,18). Alignment of the amino acid sequence surrounding the substrate site of SET8 in p53 (K382) and that of histone H4 (K20) reveals significant overlap (Fig. 2-1C). We therefore reasoned that p53 is likewise monomethylated by SET8. To confirm the extent of methylation on p53K382 catalyzed by SET8, peptides bearing amino acids 367–389 of p53 were in vitro methylated by SET8 and analyzed by mass spectrometry. As shown in Fig. 2-1D, SET8 induced a 14Da shift in the p53 peptide, indicating the addition of a single methyl group to K382. Under the conditions of our reaction, virtually all peptide was converted to the monomethyl species, with little to no dimethylation or trimethylation detected. Thus, in vitro, SET8 is a p53 monomethyltransferase.

Endogenous p53 protein is monomethylated at lysine 382. To confirm the existence of p53K382me1 in vivo, we performed a mass spectrometry analysis of endogenous p53 purified from HeLa nuclear extract (see Experimental Procedures). Two peaks representing unmethylated and monomethylated peptides containing p53K382 are shown in Fig. 2-2A. The mass difference of 14Da indicates the addition of a single methyl moiety. We confirmed by ms/ms that the methyl moiety is specific to K382 and not to other residues present in the peptide fragment (data not shown). These data provide the first definitive confirmation of methylation being present on endogenous p53.
Figure 2-2

A

endogenous p53

ms: 667
(K382LMFK)

ms: 681
(K382me1LMFK)

signal intensity

m/z

5000

4000

3000

2000

1000

0

660

665

670

675

680

685

B

peptide (ng):

p53 (aa 367-389)
p53K382me1
p53K382me2
p53K382me3
H4 (aa 1-23)
H4K20me1
H4K20me2
H4K20me3

αp53K382me1 avidin-HRP

C

p53:

WT

SET8:

- + + + + + + +

SAM:

- + + + + + + +

p53K382me1

GST-p53

GST-SET8

1 2 3 4 5 6 7 8
Characterization of a p53K382me1 modification-specific antibody

We next raised an antibody against the p53K382me1 epitope (hereafter referred to as αp53K382me1). This antibody specifically recognizes p53 peptides monomethylated at K382 and does not crossreact with unmodified p53, p53K382me2, p53K382me3 peptides or several additional p53 methylated residues (Fig. 2-2B; data not shown). Notably, despite the sequence homology between the SET8 substrate sites of p53K382 and H4K20 (see Fig. 2-1C) our antibody did not detect H4 peptides irrespective of methylation status at K20 (Fig. 2-2B). We also tested the ability of αp53K382me1 to recognize p53 methylated at K382 in the context of the full-length p53 protein. As shown in Fig. 2-2C, αp53K382me1 detects full-length SET8-methylated wild-type (wt) and various K>R mutant derivative p53 proteins (lane 4–7), but not unmethylated p53 (lane 1–3), or the p53K382R mutant (lane 8). Based on these results, we conclude that our αp53K382me1 antibody can be utilized to monitor SET8-dependent monomethylation of p53 at K382 for in vivo studies.

In vivo methylation of p53 at lysine 382. Next, to determine whether p53 is methylated by SET8 in vivo, U2OS cells were transfected with SET8 and either Flag-tagged wt-p53, p53K382R or p53K372R. As shown in Fig. 2-3A, Western analysis
detected p53K382me1 in whole cell extracts (WCE) and Flag-immunoprecipitates (IPs) from cells transfected with wt-p53 and the p53K372R mutant, but not with the p53K382R mutant. Moreover, SET8, but not a catalytically inactive mutant (SET8D338A), methylated endogenous p53 at lysine 382 in WCE and in αp53 IPs (data not shown; see Fig 2-3C; (17)). Thus, ectopic SET8 methylates endogenous p53 in vivo.

**Figure 2-3**

A schematic representation of the experimental setup is shown. The figure illustrates the detection of p53K382me1, Flag-p53, and SET8 in WCE and Flag-IPs from cells transfected with wt-p53 and the p53K372R mutant, but not with the p53K382R mutant. SET8, but not a catalytically inactive mutant (SET8D338A), methylated endogenous p53 at lysine 382 in WCE and in αp53 IPs (data not shown; see Fig 2-3C; (17)). Thus, ectopic SET8 methylates endogenous p53 in vivo.
Figure 2-3 – p53 Is monomethylated at K382 in vivo by SET8

(A) Ectopic SET8 specifically methylates p53 at K382 in vivo. Western blot analysis with αp53K382me1 antibody of Flag immunoprecipitates or WCE (whole-cell extracts) from U2OS cells expressing SET8 and the indicated Flag-tagged p53 derivatives. p53 and SET8 protein levels in WCE are shown. (B) Knockdown of endogenous SET8 decreases endogenous levels of p53K382me1 in U2OS cells. Western analysis of p53 levels present in αp53K382me1 immunoprecipitates of U2OS cells treated with control or SET8 siRNAs. Total p53, SET8, H4K20me1, and tubulin present in the WCE are shown. (C) SET8 negatively regulates acetylation of p53 at K382. Western analysis with αp53K382me1, p53K382ac, and p53 antibodies of p53 immunoprecipitates from U2OS cells transfected with control vector or SET8 and treated for 2 hr with 0.5 μg/ml NCS. SET8 and tubulin levels in the WCE are shown. Endogenous p53K382me1 is observed with longer exposure (data not shown). (D) p53K382me1 levels decrease upon DNA damage. Western analyses with the indicated antibodies of αp53K382me1 and p53 (DO1) immunoprecipitates from U2OS cells transfected with control or SET8 siRNA and treated with NCS for 2 hr. Tubulin and total p53 levels present in WCE are shown to control for loading. (E) SET8 mRNA expression decreases in response to DNA damage. Real-time PCR analysis of SET8 and Smyd2 mRNA levels present in U2OS cells ± NCS treatment (0.5 μg/ml, 4 hr). Error bars indicate ± SEM from three experiments. (F) SET8 protein levels decrease in response to DNA damage. Western analysis of SET8 in U2OS cells as in (E).
We next investigated whether SET8 is physiologically responsible for the monomethylation of p53K382. To this end, endogenous SET8 protein levels were knocked-down by RNA interference (RNAi) in the p53+ U2OS cell line, and levels of p53K382me1 determined (Fig. 2-3B). The RNAi treatment did not alter p53 levels in whole cell extracts (WCE), but levels of p53 detected in αp53K382me1 IPs were specifically reduced by SET8 RNAi relative to control RNAi treatment, denoting a decrease of endogenous p53K382me1 levels in SET8 knock-down cells (Fig. 2-3B). Three sequential transfections of SET8 RNAi are reported to result in a reduction of H4K20me1 levels (19); however, under our experimental conditions of a single round of SET8 RNAi treatment, a decrease in p53K382me1 levels is observed with no reduction in H4K20me1 levels detected (Fig. 2-3B). The manifestation of p53K382me1 depletion without reduction of H4K20me1 upon acute SET8 knock-down is likely a consequence of (i) p53 protein levels being orders of magnitude lower than H4, (ii) the rapid kinetics of p53 protein turnover and (iii) the reported high stability of the H4K20me1 mark (20). Taken together, we conclude that SET8 is required for maintenance of p53K382me1 levels in vivo.

DNA damage was reported to increase monomethylation at p53K372, suggesting the possibility that p53K382me1 might likewise be regulated (7). We therefore examined p53K382me1 levels in U2OS cells in response to the radiomimetic drug neocarzinostatin (NCS), in the presence of ectopic SET8 expression (Fig. 2-3C) and upon knock-down of endogenous SET8 protein (Fig. 2-3D). As expected, total p53 levels (and acetylation of p53 at K382 (p53K382ac)) increased in response to DNA damage induced by NCS treatment (Fig. 2-3C and 2-3D). In contrast, levels of p53K382me1 decreased in response to DNA damage compared to control treatment (Fig. 2-3C, compare lanes 3 and 4; Fig. 2-3D, compare lanes 1 and 2). Consistent with these findings, SET8 mRNA and protein expression levels in U2OS cells are markedly repressed in response to NCS treatment, whereas Smyd2 expression does not change with DNA damage (Fig. 2-3E and 2-3F). These data suggest the hypothesis that the
generation of p53K382me1 by SET8 represses p53 functions, an activity that is itself
curbed during the physiologic DNA damage response (see discussion). Finally, SET8
monomethylation at p53K382 impaired DNA damage-induced acetylation at the same
residue (p53K382ac) (Fig. 2-3C and 2-3D). The observations that p53K382me1
generation negatively correlates with DNA damage and that SET8 inhibits the
formation of p53K382ac, a modification linked to DNA damage responses, raises the
possibility that monomethylation at K382 inhibits p53-mediated DNA damage
responses (5,6).

SET8 suppresses p53-dependent transcription activation. To investigate the
biological consequences for SET8-mediated methylation of p53, the ability of SET8 to
regulate transcription of the p53 target genes p21 and PUMA was determined. For
these experiments, we used H1299 cells, which lack endogenous p53 and can
therefore be complemented with either wild-type or mutant p53, allowing for the
selective investigation of the role of K382 (21,22). p53 reconstitution in H1299 cells
resulted in marked induction of p21 mRNA (Fig. 2-4A) and p21 protein (Fig. 2-4B)
levels relative to cells transfected with vector alone. The p53K382R mutant likewise
triggered a strong induction of the p53 target genes, though the phenotype was slightly
less pronounced than wild-type p53, indicating that K382 residue per se is not
required for p53 transactivation activity (Figs. 2-4A and 2-4B; data not shown).
Notably, co-expression of SET8 with p53, which did not affect p53 protein
expression, largely abolished the induction of p21 mRNA and p21 protein elicited by
wt-p53, yet did not impinge on the activity of the p53K382R mutant (Figs. 2-4A and
2-4B). Equivalent results were obtained for PUMA, a second p53 target gene (data not
shown). Additionally, we observed that in chromatin immunoprecipitation (ChIP)
assays, SET8 co-expression reduced occupancy of p53 at the p21 and PUMA
promoters (Fig. 2-4C; data not shown), but notably had no effect on H4K20me1 levels
at these promoters (Fig. 2-4D; data not shown). p53K382me1 was not detected at the
p21 and PUMA promoters, though global distribution of this species is grossly similar
to that of total p53 (data not shown). Finally, the SET8 catalytic mutant SET8D338A,
which fails to methylate p53 in vivo, likewise failed to inhibit p53 transactivation.
activity (Fig. 2-4E, data not shown). Taken together, these data argue for a model in which it is the direct addition of the methyl moiety to K382 by SET8, rather than alterations of H4K20me1 levels by SET8 or the failure to otherwise modify p53K382, which acts to inhibit p53 transcriptional activity.

**Figure 2-4**
Figure 2-4. SET8 methylation of p53 at K382 suppresses p53 transactivation activity

(A and B) SET8 inhibits induction of p21 transcription (A) and p21 protein expression (B) by WT p53 but does not affect the activity of the p53K382R mutant. (A) Real-time PCR analyses of p21 mRNA levels in H1299 cells transfected with control vector, p53, or p53K382R mutant, ± SET8. (B) Western analyses with the indicated antibodies of H1299 cell WCE as in (A). (C) SET8 expression attenuates occupancy of p53 at the p21 promoter. p53 occupancy at the p21 promoter in H1299 cells transfected with control vector or p53, ± SET8, was determined by ChIP analyses. DO1 antibody was used for p53 ChIP, and IgG was used as control. Occupancy values (ChIP/input × 100) were determined by real-time PCR. (D) SET8 expression does not alter H4K20me1 levels at the p21 promoter. Occupancy of H4K20me1 (H4K20me1 ChIP/H3 ChIP × 100) at the p21 promoter was determined as in (C). Error bars in (A), (C), and (D) indicate ± SEM from three experiments. (E) SET8 catalytic mutant SETD338A fails to suppress p53 transactivation activity on target genes. Real-time PCR analyses of relative p21 mRNA levels in H1299 cells cotransfected with p53, SET8, SET8D338A, or control vector as indicated. Error bars indicate ± SEM of triplicate repeats from two independent experiments.

To test this hypothesis in a more physiologic setting, endogenous SET8 protein levels were knocked-down in the p53+ U2OS cell line, and p53-dependent responses to NCS treatment were determined. Consistent with the overexpression data, knockdown of SET8 led to an increase of p21 mRNA levels and p21 protein expression induced by the genotoxic agent relative to the control siRNA treatment (Fig. 2-5A and 2-5B). For these experiments, two independent siRNA pools were utilized to exclude off-target effects (Fig. 2-5A and 2-5B). Like p21, the expression of PUMA was upregulated by SET8 RNAi treatment relative to control treatment (data not shown).
Figure 2-5 – SET8 RNAi augments p53 activity in response to DNA damage

(A and B) Knockdown of SET8 augments expression of p21 mRNA (A) and p21 protein (B) in response to DNA damage. (A) Real-time PCR analyses of p21 mRNA in U2OS cells treated with 0.5 µg/ml NCS (4 hr) and transfected with control or two different sets of SET8 siRNA. (B) Western analyses with the indicated proteins of WCE from U2OS cells as in (A). Both SET8 RNAi sets depleted endogenous SET8 protein levels without altering H4K20me1 levels. H4 levels are shown to control for equal loading. (C) Real-time PCR analysis of p53 mRNA in U2OS cells transfected with control or SET8 siRNA ± p53 siRNA under normal condition or NCS treatments. (D) Western analysis of p53 in U2OS cells transfected with control or p53 siRNA. Tubulin levels are shown to control for loading. (E) SET8 regulation of p21 expression is p53 dependent. Real-time PCR analysis of p21 mRNA in U2OS cells as in (C). (F) Knockdown of endogenous SET8 augments p53 occupancy at the p21 promoter. ChIP assays as in Fig. 2-4C in U2OS cells transfected with control or SET8 siRNA ± NCS treatment. (G) SET8 RNAi does not alter H4K20me1 levels at the p21 promoter. ChIP assays to determine H4K20me1 occupancy at the p21 promoter as in Fig. 2-4D in U2OS cells as in (F). Error bars in (A), (C), and (E)–(G) indicate ± SEM from at least three experiments.

It is possible that the altered regulation of p21 and PUMA in response to DNA damage by SET8 knock-down is due to modulation of H4K20 methylation rather than p53 methylation. However, this possibility is highly unlikely for a number of reasons: (1) irrespective of the different treatments of our experiments, no SET8-dependent changes in H4K20me1 levels were observed globally (Fig. 2-5B) or at specific promoters (Fig. 2-5G; data not shown); (2) the increase in p21 and PUMA induction upon DNA damage observed with SET8 knock-down is dependent on p53 protein, as co-knock-down of p53 (Fig. 2-5C and 2-5D) eliminated any effects of SET8 (Fig. 2-5E; data not shown); (12) the SET8 knock-down mediated increase in gene expression of p21 and PUMA correlates with increased p53 occupancy at the cognate promoters, indicating SET8 directly alters p53 behavior (Fig. 2-5F; data not shown); (4) the
regulation of highly responsive p53 target genes is specific, as expression of a constitutively active non-p53 regulated gene (actin) did not change upon SET8 knockdown, as might be expected were the phenotype due to global loss of H4K20me1 (data not shown); (5) knock-down of SET8 did not impact the expression of a number of weak p53 targets such as Bax and NOXA, and suppressed mRNA induction of the DNA repair factor GADD45, the inverse of what would be expected were the phenotype due to H4K20me1 depletion (data not shown). Together, these observations strongly argue for direct methylation by SET8 at lysine 382 of p53 – and not lysine 20 of histone H4 – in the regulation of p53 responses.

**SET8 depletion augments p53 cellular functions.** We next addressed the role of SET8 in p53-dependent cell-cycle arrest and apoptosis. In the absence of DNA damage and consistent with a previous report (15), SET8 knock-down cells behave like control RNAi cells with respect to cell-cycle progression (Fig. 2-6A). In contrast, upon DNA damage, SET8 knock-down renders cells more prone to apoptosis relative to control cells (9.9% Sub-G1 fraction in SET8 RNAi cells versus 3.1% for control cells; Fig. 2-6A). Further, SET8 RNAi cells exhibit a higher fraction of cells in G1 than control cells (56.5% versus 38%, respectively; Fig. 2-6A). These effects are strictly dependent on p53, as co-knock-down of p53 (see Fig. 2-5D) eliminates the DNA damage-dependent phenotype of SET8 knock-down cells (Fig. 2-6B). To further test whether the increased sensitivity of SET8 knock-down cells to DNA damage is mediated by p53, cell death induced by the DNA damage agent doxorubicin was also determined in the absence or presence of both SET8 and p53 knock-down. As shown in Fig. 2-6C, whereas SET8 RNAi alone increases sensitivity of cells to DNA damage relative to control, this effect is abolished in a p53 knock-down background.
Figure 2-6

A. Control siRNA vs. SET8 siRNA
   - Dox
   - DNA content
   - Sub-G1: 1.6% vs. 0.3%
   - G1: 48.7% vs. 46.3%
   - S: 23.2% vs. 21.3%
   - G2/M: 26.5% vs. 31.4%

B. p53 siRNA vs. p53 siRNA + SET8 siRNA
   - DNA content
   - Dox
   - Sub-G1: 0.3% vs. 0.3%
   - G1: 59.8% vs. 51.9%
   - S: 19.1% vs. 23.9%
   - G2/M: 20.8% vs. 23.9%

C. % cell death
   - control siRNA: + - - -
   - SET8 siRNA: - + - +
   - p53 siRNA: - - + +
Figure 6. Monomethylation of p53 at K382 attenuates p53 biological function

(A) SET8 knockdown renders cells more sensitive to cell death and cell-cycle arrest following DNA damage. Sub-G1 and cell-cycle distribution of U2OS cells ± SET8 siRNA and ± 24 hr treatment with 1 µg/ml doxorubicin was determined by flow cytometry. (B) The increased sensitivity of SET8 knockdown cells to DNA damage is p53 dependent. Cell-cycle distribution of SET8 knockdown cells as in (A) ± p53 knockdown. (C) Increased sensitivity of SET8 knockdown cells to DNA damage-induced cell death is p53 dependent. Cell death was determined in U2OS cells ± SET8 siRNA and ± p53 siRNA, in response to 2 µg/ml doxorubicin for 20 hr. Error bars indicate ± SEM from at least three experiments.

Discussion

Here we have shown that the HMT enzyme SET8/PR-Set7 specifically monomethylates p53 at lysine 382 in vitro and in vivo. We have also provided the first mass spectrometry evidence that endogenous p53 is methylated (see Fig. 2-2A). Ectopic expression of SET8 suppresses p53 transactivation activity and knock-down of endogenous SET8 by RNAi augments a number of p53 activities, including induction of highly responsive target genes, and increased apoptosis and cell-cycle arrest (see model, Fig. 2-7). Numerous experiments indicate that these phenotypes are a consequence of SET8 methylation of p53, and not an indirect effect of H4K20 methylation by SET8. For example, in H1299 cells, SET8 represses ectopic p53, but fails to do so if p53 harbors a mutation at K382 (p53K382R) (Figs. 2-4A and 2-4B). If SET8 repression of p53 target genes occurred via H4K20 methylation rather than p53K382 methylation, then the transactivation activity of wild-type and mutant p53K382R proteins should both be equally affected by SET8. As we do observe a requirement for K382 to be intact, we conclude that availability of this residue for methylation by SET8 is needed for SET8 to repress p53. Further evidence for a direct regulation of p53 by SET8 is that all SET8 RNAi phenotypes associated with DNA damage (e.g. increased p21 induction and cell death) are absolutely dependent on the
presence of p53 protein. Thus, SET8, like a number of other p53-regulatory enzymes, such as TIP60, SIRT1, SET7/9 and HDAC1, utilizes both p53 and histones as substrates – with distinct functions specific to the different substrates (23,24) (7,11,25-28).

Figure 2-7
Figure 2-7. Model for SET8 regulation of p53

Under normal conditions, a population of p53 is monomethylated at K382 by SET8, which might render p53 inert in part by preventing acetylation at K382. Upon DNA damage, the inhibitory effect of p53K382me1 might be reversed by a combination of SET8 downregulation being coupled to increased p53 stability, and potentially via methylation of p53K382me1 to p53K382me2/3 by an as yet unknown histone methyltransferase (HMT) and/or demethylation by an as yet unknown demethylase (HDM).

We observe that p53K382me1 levels decrease with DNA damage. This observation, in conjunction with our functional characterization, argues that SET8 is a negative regulator of p53 activity. However, we do observe a SET8-dependent upregulation of the DNA repair gene GADD45, indicating that p53K382 methylation might have more complex regulatory roles than simply contributing to on/off functions. In this regard, under normal conditions, monomethylation at K382 might render p53 largely inert but predisposed to respond to specific cellular stresses (Fig. 2-7). Alternatively, this methylation event might dampen p53 as a mechanism for measured responses to mild insults, allowing for p53-dependent repair of DNA without the induction of cell death.

At the molecular level, how might K382 monomethylation be coupled to regulation of p53 functions? We postulate that the biological consequences of p53K382me1, alone, or in the context of additional modifications, might be dictated by distinct protein binding partners; different proteins can therefore define and channel p53 towards divergent activities. In this context, the MBT domain is a protein module found commonly on nuclear proteins and that has been shown to bind monomethyllysines (29), raising the possibility that a modular interaction between an MBT domain-containing protein and p53K382me1 acts to repress p53. There are several other methyllysine-binding domains that preferentially recognize higher methyl states than monomethyllysine (3,30,31). This raises the question of whether SET8 monomethylation of p53 at K382 leaves p53 poised to be further methylated in
response to a specific signal, and that the higher methyl forms are linked to additional p53 functions (Figure 7). We have not detected methyltransferase activity on p53K382 by Suv4-20h1 and Suv4-20h2, two well-characterized H4K20 HMTs (X. Shi and O. Gozani, unpublished observations) (32). In addition to these two enzymes, there are other putative H4K20 HMTs, such as ASH1 and NSD1, as well as numerous uncharacterized SET domain containing proteins, and perhaps one of these functions as a di- or trimethyltransferase at p53K382 (33,34). In summary, this study reveals novel functions for the SET8 HMT in regulating the non-histone protein p53, and supports the hypothesis that protein lysine methylation likely modulates diverse cellular processes (35).

Materials and Methods

Plasmids, Antibodies and Peptides – SET8 cDNA was cloned into pcDNA3.1. p53 cDNA was cloned into pCAG-Flag and pGEX6p. SET8 and p53 mutants were generated by site-direct mutagenesis (Stratagene). Primer sequences are available upon request. αp53K382me1 antibody was generated in rabbits immunized with the monomethylated peptide: 377-TSRHKK(me)LMFKT-387. Other antibodies used in this study are: HRP-p53 (R&D systems); p53 (DO1) and p21 (EA10) (Calbiochem); p53K382ac (Cell Signaling); SET8, H4K20me1 and H3 (Abcam); p53(FL-393) and GST (E5) (Santa Cruz); Flag (M2) and tubulin (Sigma). p53 and histone peptides bearing different modifications were synthesized at the W.M. Keck Facility at Yale.

Cell Culture and Transfections – U2OS, H1299 and 293T were maintained in DMEM medium supplemented with 10% fetal bovine serum. Cells were transfected with plasmids or siRNA duplexes by TransIT-LT1 (Mirus) or DharmaFECT (Dharmacon), respectively, according to the manufacturers’ protocols.

Immunoprecipitation and Western Blots – Endogenous p53 or ectopically expressed Flag-tagged p53 were IPed with agarose conjugated p53 or Flag antibodies from whole cell extracts in cell lysis buffer (50 mM TrisHCl pH7.4, 250 mM NaCl, 0.5% Triton X100, 10% glycerol, 1 mM DTT, 1mM PMSF and protease inhibitors). After
incubation at 4°C for overnight, the beads were washed 3X with the same buffer, and boiled in 2xLaemmli buffers. The IPed p53 was resolved on SDS-PAGE gel and detected by antibodies against state-specific p53 antibodies or HRP-αp53 to avoid crossreactivity with IgG heavy chain.

*In Vitro methyltransferase assay* – Methyltransferase assays were performed as previously described (36). Briefly, 2 μg of GST-p53, bulk histones, nucleosomes, or 1 μg of p53 or H4 peptides were incubated with 1μg of recombinant HMT and 0.1 mM S-adenosyl-methionine (SAM, Sigma), or 2 μCi 3H-SAM Amersham) in reaction buffer containing 50mM Tris-HCl pH 8.0, 10% glycerol, 20mM KCl, 5 mM MgCl₂, 1 mM DTT and 1 mM PMSF, at 30°C for 30 min to 2hrs. The reaction mixtures were then subject to electrophoresis on SDS-PAGE, followed by either radiography or Westerns. The reactions with peptides were subject to mass spectrometry analysis.

*Protein Purification and Mass Spectrometry* – Hela nuclear extracts were prepared as previously described (37). To IP endogenous p53 proteins, ~10 mg of nuclear extracts were incubated with 50 μl of DO1-conjugate agarose in buffer containing 20 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.01% SDS, 1% Trition X 100, 1 mM EDTA and protease inhibitors with gentle rotation at 4°C for overnight. The beads were washed 2X with the same buffer, 2X with high salt buffer (20 mM Tris-Cl pH 8.0, 500 mM NaCl, 0.1% SDS, 1% Trition X 100, 2 mM EDTA), once with LiCl buffer (20 mM Tris, pH 8.0, 500 mM LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA), and once with TE buffer (10 mM TrisHCl 8.0, 1 mM EDTA). The p53 protein bound to the beads was subject to SDS-PAGE, excised from the gels, and incubated with trypsin overnight at 37 C. Pooled supernatants containing extracted peptides were dried and resuspended in 30% acetonitrile and 0.1% TFA prior to mass spectrometry analysis. Samples were analyzed on a reflectron time-of-flight mass spectrometer, MALDI-TOF instrument (Ultraflex, Bruker Daltonics, Billerica, MA), equipped with a 337 nm nitrogen laser and delayed ion extraction capability (delay times: 30–50 ns). Ion structure information was obtained by post source decay (PSD), using the mass gate feature, to select specific m/z window for fragmentation. The mass gate resolution was 1% of the precursor mass. Data was recorded in both positive and negative ion modes.

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at 20kV acceleration, and mass analysis of ions determined using a dual micro-channel plate detector. Detector output was collected with a 1 GHz digitizer and displayed directly on a Windows NT based computer. Ten positive ion reflectron TOF mass spectra of 1000 laser shots were accumulated and externally calibrated with commercial peptide mix (Bruker Daltonics, Billerica, MA). For analysis of in vitro methylated synthetic peptides, the synthetic peptides untreated and treated with SET8 were equilibrated with 0.1% trifluoroacetic acid (TFA), and 50% acetonitrile with 0.1% TFA, and applied to the MALDI target plate with equal volumes of the matrix α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma).

**siRNA-Mediated Knockdown of SET8 and p53** – The knockdown of SET8 was performed by transfection of U2OS cells for 48 hrs with two sets of Dharmacon on-target plus siRNA duplex targeting human SET8 (5′-AGUCAAAAGAUCUAUUCCAUUU-3′/5′-GCAACUAGAGAGACAAACUUU-3′) or (5′-GGAAACCAUUAGCCGAAUUU-3′/5′-GUACGGAGCGCCAUGAAGUUU-3′) respectively, or with on-target plus SMARTpool SET8 siRNA, by using DharmaFECT according to the manufacturer’s protocol. p53 knockdown was carried out with Dharmacon on-target plus p53 siRNAs (5′-GAAAUUUGCGUGUGAGUAAU-U3′/GUGCAGCUGUGGGUUGAUUUU-3′). On-target plus siControl siRNA (5′-UGGUUUACAUGUCGACUAA-3′, Dharmacon) or on-target plus SMARTpool siControl siRNA were used as controls.

**RT-PCR, Real-time PCR and ChIP Assays** – RT-PCR and real-time PCR and ChIP assays were performed as previously described (36). Cells were treated with NCS (0.5 µg/ml, Sigma) for 2 to 4 hrs. mRNA was prepared using RNeasy plus kit (Qiagen) and reverse-transcribed using First Strand Synthesis kit (Invitrogen). Quantitative Real-time RT-PCR was performed on the ABI PRISM 7700 Sequence Detection System using Taqman Gene Expression Assay primer/probe sets (Applied Biosystems). Gene expressions were calculated following normalization to GAPDH levels by the comparative Ct (Cycle threshold) method. Primer sequences used for ChIP analyses are: p21 promoter: 5′-GTGGCTCTGATTGGCTTT-CTG-3′/5′-CTGAAAACAGGCAGCCCAAAG-3′. PUMA promoter: 5′-
GCGAGACTGTGGCCTTGTGT-3′/5′-CGTTCCAGGGTCCACAAAGT-3′. Other primer sequences are available upon request.

**Cell Cycle and Cell Death Assays** – For flow cytometry, U2OS cells were collected and fixed with 70% ethanol at 4°C for 4 hrs to overnight. Cells were then washed with PBS for 3 times and stained with 10 μg/ml propidium iodide and 100 μg/ml RNase. Cell death assays were performed as previously described (28,36). Briefly, following treatment, cells were stained with trypan blue and the fraction of cells uptaking the dye was determined utilizing a hemocytometer.

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**References**


Chapter Three: The MBT repeats of L3MBTL1 link SET8 mediated p53 methylation at lysine 382 to target gene repression

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Summary

The p53 tumor suppressor protein is regulated by multiple post-translational modifications, including lysine methylation. We previously found that monomethylation of p53 at lysine 382 (p53K382me1) by the protein lysine methyltransferase (PKMT) SET8/PR-Set7 represses p53 transactivation of target genes. However, the molecular mechanism linking p53K382 monomethylation to repression is not known. Here we show in biochemical and crystallographic studies the preferential recognition of p53K382me1 by the triple malignant brain tumor (MBT)-repeats of the chromatin compaction factor L3MBTL1. We demonstrate that SET8-mediated methylation of p53 at K382 promotes the interaction between L3MBTL1 and p53 in cells, and the chromatin occupancy of L3MBTL1 at p53 target promoters. In the absence of DNA damage, L3MBTL1 interacts with p53K382me1 and p53-target genes are repressed, whereas depletion of L3MBTL1 results in a p53-dependent increase in p21 and PUMA transcript levels. Activation of p53 by DNA damage is coupled to a decrease in p53K382me1 levels, abrogation of the L3MBTL1-p53 interaction, and disassociation of L3MBTL1 from p53-target promoters. Together, we identify L3MBTL1 as the second known methyl-p53 effector protein, and provide a molecular explanation for the mechanism by which p53K382me1 is transduced to regulate p53 activity.
Introduction

The reversible and dynamic methylation of proteins on the nitrogen side-chain of lysine residues can greatly increase the signaling potential of the modified factor [1, 2]. Lysine residues can accept up to three methyl groups, forming mono-, di- and trimethylated derivatives, with a unique activity frequently being coupled to the specific methylation state. The chemical addition of methyl moieties to histones is not believed to intrinsically affect chromatin structure. Rather, the principal mechanism by which histone lysine methylation is thought to manifest functionally occurs through regulation of modular protein-protein interactions [3-5]. In this regard, the protein(s) that recognize a methylated lysine within a specific sequence context can define the functional outcome associated with that specific lysine methylation event. Thus, mechanistic insight into how lysine methylation influences a biological program requires knowledge of the proteins and domains that recognize and transduce this modification.

In addition to histones, several other proteins such as the tumor suppressor p53 undergo lysine methylation, arguing that this modification may be a common mechanism for modulating protein-protein interactions and key cellular signaling pathways [6-8]. p53 plays a pivotal role in the regulation of cellular responses to various forms of genotoxic stresses, and its activity is coordinated by a complex network of post-translational modifications [9, 10]. We previously demonstrated that the protein lysine methyltransferase PR-Set7/SET8 monomethylates p53 exclusively at lysine 382 (p53K382me1), and that the placement of this modification negatively regulates p53 activity [11]. Specifically, SET8 suppresses transcriptional activation of p53 rapid response target genes [11]; however, the molecular explanation for how p53K382me1 is sensed and transduced to represses p53 is not clear.

In this study we sought to understand how monomethylation of p53 at K382 negatively regulates p53 function. Recently, p53 transcriptional activity was shown to be restrained at chromatin via an interaction with Cabin1, suggesting that a general mechanism for modulating p53 function might occur at the level of chromatin regulation [12]. L3MBTL1 represses transcription by promoting a more compact,
inaccessible chromatin state [13-15]. This chromatin compaction activity requires the three MBT domains of L3MBTL1, with the middle MBT domain functioning as a binding module of mono- and di- methylated histone lysines that are mostly enriched at silenced chromatin (e.g. H4K20me1/2 and H1K26me1/2) [13, 16-21]. Here we have identified that L3MBTL1, via its MBT motifs, binds to p53K382me1. We provide evidence for a model by which L3MBTL1 acts as a dual methyllysine-sensor, coupling the methylated form of p53 to silenced histone methyl marks to render p53 inert at target genes.

Results

The 3xMBT repeat of L3MBTL1 recognizes p53K382me1 in vitro. L3MBTL1 binds to H4K20me1 through its second MBT repeat [17, 18, 20, 21]. Considering the sequence similarity between H4K20 and p53K382 (Fig. 3-1A), we tested if L3MBTL1, through the triple MBT domain, recognizes p53K382me1. In vitro peptide pull-down assays were performed with recombinant GST-tagged 3xMBT (L3MBTL13xMBT) repeats and biotinylated p53 peptides (amino acids 367-389) in which K382 was unmodified or modified by mono-, di-, or tri-methylation: p53K382me1, p53K382me2, or p53K382me3 respectively. As shown in Figs. 3-1B and 3-1C, L3MBTL13xMBT bound preferentially to p53K382me1 and p532K382me2 peptides relative to unmodified or trimethylated peptides, similar to its known methyl state binding preference for H4K20. Finally, the binding affinity of L3MBTL13xMBT for p53K382me1 and p53K382me2 peptides was comparable to its affinity for H4K20me1, as determined by isothermal calorimetry (Fig. 3-1C) [17, 18, 20, 21]. Based on these data, we conclude that the 3xMBT domain of L3MBTL1 binds in vitro to p53K382me1.
Figure 3-1

A

H4 1 - 24: SGRGKGGKGLGGAKRHRKVL-R---
p53 367 - 389: S-HLKSKKG---QSTSRHKKLMFKTEG
* H4K20

B

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Kd (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 (aa 377-386)</td>
<td>N.B.</td>
</tr>
<tr>
<td>p53K382me1</td>
<td>28 ± 5 µM</td>
</tr>
<tr>
<td>p53K382me2</td>
<td>24 ± 6 µM</td>
</tr>
<tr>
<td>p53K382me3</td>
<td>N.B.</td>
</tr>
<tr>
<td>H4 (aa 1-23)</td>
<td>N.B.</td>
</tr>
<tr>
<td>H4K20me1</td>
<td>20 ± 1 µM</td>
</tr>
</tbody>
</table>

C

D

E

F
Figure 3-1 – L3MBTL1\textsubscript{(3xMBT)} recognizes p53 monomethylated at lysine 382 (p53K382me1) \textit{in vitro}

\textbf{(A)} Sequence alignment of the H4 N-terminus and p53 C-terminal tail. Homology surrounding the SET8/53BP1 binding site is highlighted. Asterisks indicate methylation sites. \textbf{(B)} L3MBTL1\textsubscript{(3xMBT)} binds to p53K382me1 and p53K382me2 peptides. Shown are the results from Western analysis of peptide pull-down assays with the indicated biotinylated peptides and GST-L3MBTL1\textsubscript{(3xMBT)}. \textbf{(C)} Dissociation constants (K\textsubscript{d}) of p53 or histone H4 peptides for L3MBTL1\textsubscript{(3xMBT)} were determined by ITC. K\textsubscript{d} of H4K20me1 is shown as control. \textbf{(D)} The L3MBTL1\textsubscript{(3xMBT)} repeats are shown as a solid surface with the residues comprising the p53K382me1-binding pocket of MBT2 labeled and colored orange, brown and yellow for aromatic, acidic and hydrophobic/polar residues, respectively. Lysine 382me1 of the p53K382me1 peptide is shown as a stick model with C, O and N atoms colored green, red and blue, respectively. The unoccupied semi-aromatic pockets MBT1 and MBT3 are colored gray. \textbf{(E)} Close-up view of the K382me1-binding cage. A dotted red line depicts the hydrogen bond between the methylammonium proton and the carboxyl group of D355. \textbf{(F)} Point mutations in the MBT2 repeat of L3MBTL1 abolish the p53K382me1-binding activity of L3MBTL1\textsubscript{(3xMBT)} \textit{in vitro}. Western analysis of the indicated GST-L3MBTL1\textsubscript{(3xMBT)} mutants in peptide pull down assays are shown.

\textit{Structural basis of L3MBTL1 recognition of p53K382me1.} To gain insight into the p53K382me1 – L3MBTL1 interaction at the molecular level we solved the crystal structure of L3MBTL1\textsubscript{3xMBT} bound to the p53K382me1 peptide. The crystal structure of the 3xMBT repeats in complex with a peptide corresponding to residues 377-387 of p53 monomethylated at K382 was determined at a 2.5 Å resolution. The protein structure comprises three globular domains arranged into a three-leaved propeller-like fold (Fig. 3-1D and data not shown). Each MBT repeat is composed of two subunits.
The N-terminal subunit contains short helices and long loops, whereas the C-terminal subunit consists of four anti-parallel β-strands folded into a barrel-like shape. The N-terminal subunit of each repeat packs against the C-terminal subunit of the neighboring repeat resulting in an arrangement in which the amino- and carboxy-termini of 3xMBT are nearby. The overall structure of the protein is in agreement with the previously reported structures of L3MBTL13xMBT [20, 21, 27].

Of the three molecules comprising an asymmetric unit, two were complexes of 3xMBT with the p53K382me1 peptide (chains A and C) and one was the ligand-free protein (chain B). The structure of p53K382me1-bound 3xMBT superimposes well with the structure of the unbound protein (root mean square deviation of 0.3/0.4 Å between chain B and chains A/C, over Cα atoms), indicating that binding to the p53K382me1 peptide does not induce significant conformational changes. In the complex (chains A and C), the p53K382me1 peptide was bound by the second MBT domain only (MBT2), despite the fact that all three repeats have conserved amino acid sequences and structures, which was also observed for the interaction of L3MBTL13xMBT with mono- and di-methylated histone peptides [20, 21]. Although the electron density was not seen for most of the peptide residues, it was clear for K382me1. The fully extended side chain of K382me1 inserted almost perpendicularly to the protein surface into a deep cavity created by aromatic F379, W382 and Y386 residues, hydrophobic/polar N358, L361, C363 and T411 residues, and an acidic D355 (Fig. 3-1E). The carboxylate of D355 forms a hydrogen bond with one of protons of the methylammonium group as well as a salt bridge with this positively charged moiety. A close comparison of the bound and unbound states (chains C and B) reveals that the binding pocket is pre-formed, with each protein residue occupying essentially the same position in both states (data not shown). This binding mode is similar to the mechanism by which L3MBTL13xMBT, Tudor domain and engineered plant homeodomain (PHD) fingers, recognize mono- and di-methylated lysine residues of histones [16, 20, 28], suggesting conservation for readout of the low-methylation state.

To test the importance of the MBT2 binding pocket in recognition of p53K382me1, a mutational analysis of the MBT pockets was performed. As shown in
Fig. 3-1F, mutation of any of the individual residues implicated in the structure analysis to be important for MBT2 binding to p53K382me1, abrogated p53K382me1-recognition. In contrast, analogous mutations within the MBT1 and MBT3 repeats did not affect binding to p53K382me1 (data not shown). Taken together, we conclude that the second MBT repeat of L3MBTL1 binds to p53K382me1 with a molecular mode of action similar to L3MBTL1 recognition of H4K20me1.

*SET8*-mediated p53K382 methylation augments L3MBTL1 binding to p53 in cells. Next, the importance for p53 methylation in mediating p53 – L3MBTL1 interaction in cells was investigated. We overexpressed Flag-tagged L3MBTL1 with or without SET8 in 293T cells for coimmunoprecipitation (co-IP) assays. As shown in Fig. 3-2A far more endogenous p53 was co-IPed in cells co-transfected with SET8 and L3MBTL1 relative to L3MBTL1 alone (compare lanes 3 and 4), suggesting that monomethylation of p53 at K382 augments the ability of L3MBTL1 to bind to p53 in cells.
Figure 3-2 – L3MBTL1 binds to p53K382me1 in vivo

(A) SET8 augments the interaction between p53 and L3MBTL1 in vivo. Western analysis with the indicated antibodies of the Flag IPs or whole cell extract (WCE) from 293T cells expressing the indicated proteins is shown. Tubulin levels in the WCE are shown as loading control. (B) Monomethylation on p53K382 by SET8 is important for the association of L3MBTL1 and p53 in vivo. Western analysis with the indicated antibodies of endogenous L3MBTL1 IPs or WCE from H1299 cells expressing wild-type p53 or p53K382R mutant in the presence of SET8 or SET8(D338A) as indicated.
Tubulin is shown as a loading control. (C) The intact MBT2 domain is required for interaction between L3MBTL1 and p53 in vivo. Western analysis with the indicated antibodies of the Flag IPs or WCE from 293T cells expressing wild-type F-L3MBTL1 or F-L3MBTL1(D355N) mutant ± SET8. Tubulin levels in the WCE are shown as loading control.

To specifically address the role of p53K382 methylation in the p53-L3MBTL1 interaction, we performed co-IPs in the p53 null strain H1299 reconstituted for p53 with either wild-type (wt) p53 or a p53K382R mutant that cannot be methylated by SET8. SET8 or a SET8 catalytic mutant (SET8D338A) [29, 30] were cotransfected with the wt or mutant p53 into H1299 cells and endogenous L3MBTL1 was IPed. As shown in Fig. 3-2B, p53 was clearly present in the L3MBTL1 IP in cells cotransfected with wt p53 and wt SET8, whereas in all other transfection combinations, p53 was not detected in the L3MBTL1 IP. These results argue that SET8 monomethylation of p53 at lysine 382 is important for in vivo interaction of p53 with L3MBTL1.

To further investigate the importance of p53K382 methylation and its recognition by MBT2 of L3MBTL1 in the L3MBTL1-p53 interaction, the ability of L3MBTL1 or a L3MBTL1(D355N) mutant, shown to disrupt binding to p53K382me1 in vitro (Fig. 3-1), to co-IP endogenous p53 in a SET8-dependent fashion was determined. The increase in interaction between L3MBTL1 and p53 induced by SET8 was diminished in the L3MBTL1(D355N) mutant IP (Fig. 3-2C). We note that SET8 does facilitate binding between L3MBTL1 and p53 in a methylation-independent fashion, potentially via formation of a trimeric complex (data not shown). Together, we conclude that recognition of p53K382me1 by the second MBT repeat of L3MBTL1 is important for L3MBTL1-p53 interactions in cells.

L3MBTL1 represses p53 target genes at basal conditions. We previously demonstrated that induction of p53 target genes, such as p21, is negatively regulated by SET8-mediated monomethylation at p53K382 under both basal conditions and in response to DNA damage [11]. L3MBTL1 has been implicated in chromatin compaction and promotion of a repressive chromatin state [13, 15]. We therefore
postulated that the repressive effect of p53K382me1 at these promoters might in part be due to binding to and stabilizing L3MBTL1 at p53 target genes. To test this hypothesis, we determined in chromatin immunoprecipitation (ChIP) assays the occupancy of stably expressing Flag-tagged L3MBTL1 (introduced by retroviral transduction (Fig. 3-3A)), endogenous p53 and H4K20me1 at the p21 and PUMA genes in wild-type HCT116 cells and p53−/− HCT116 cells [31]. As shown in Fig. 3-3B, both p53 and L3MBTL1 occupy the p21 and PUMA (data not shown) promoter under basal conditions, but are not detected in the p21 and PUMA transcribed body. Moreover, in p53−/− cells, L3MBTL1 is not detected at the two promoters (Fig. 3-3B; data now shown). H4K20me1, which is generated by SET8 [29, 30] and can also be bound by L3MBTL1 [13], is detected at low levels at promoters and does not show a difference in signal between p53+/+ and p53−/− cells (Fig. 3-3B; data not shown.) Together, these data suggest that p53 can play a role in stabilizing L3MBTL1 at its target promoters.
Figure 3-3

A

B

C

D

E

57
Figure 3-3 – L3MBTL1 represses p53 target genes under normal conditions

(A) Western blot with L3MBTL1 antibodies of WCE from the indicated cell lines transduced with control, Flag-L3MBTL1, or Flag-L3MBTL1-D355N retrovirus. (B) p53 stabilizes L3MBTL1 at target genes under basal conditions. Chromatin immunoprecipitation (ChIP) assays to determine occupancy at the promoter and 3’ transcribed body region of the p21 gene in wt and p53–/– HCT116 cells stably expressing Flag-L3MBTL1 or Flag-L3MBTL1-D355N as indicated. ChIPs: Flag (left); p53 (middle); H4K20me1 (right). Occupancy values (% input) were determined by real-time PCR. (C) p21 expression increases upon L3MBTL1 depletion in the presence of endogenous p53 and in the absence of DNA damage. U2OS or the p53 negative cell line H1299 treated with control or two independent L3MBTL1 siRNAs and L3MBTL1 and p21 mRNA levels were determined by real-time PCR. (D) L3MBTL1 depletion attenuates cellular proliferation rates. Left panel: 7-day growth curve time-course for U2OS cells stably expressing control or two independent L3MBTL1 shRNAs. Error bars indicate standard error of the mean (S.E.M.) from three experiments. Right panel: Real-time PCR of L3MBTL1 mRNA levels of cells used in the growth curve experiment. (E) L3MBTL1 Depletion attenuates colony growth. Left panel: Colony formation panel of cells as in (D). Representative plates are shown for control and the two L3MBTL1 stable knockdown lines. Right panel: Quantitation of colonies counted for each cell line. Error bars indicate S.E.M. from three independent experiments.

Next, the role of L3MBTL1 in regulating p21 mRNA expression was determined. Endogenous L3MBTL1 was depleted by RNA interference employing two independent L3MBTL1-targeting siRNAs. As shown in Fig. 3-3C, in the absence of DNA damage (a condition that stimulates p21 expression), depletion of L3MBTL1 induced an approximately 2-fold increase in p21 transcription in the p53 positive U2OS cells. In contrast, no up-regulation of p21 transcript was observed upon L3MBTL1 knock-down in p53 negative H1299 cells. Together, these results suggest that (1) L3MBTL1 normally plays a role in maintaining p21 repressed, (2) this
repression is p53 dependent, and (3) the molecular mechanism for this repression is in part due to p53 stabilization of L3MBTL1 at chromatin of p53 target genes.

Stable depletion of L3MBTL1 in U2OS cells with two independent shRNAs resulted in markedly slower cell proliferation rate relative to cells expressing the control shRNA (Fig. 3-3D). Moreover, colony formation assays indicated that fewer and smaller colonies develop in L3MBTL1 depleted cells relative to control cells (Fig. 3-3E). These data suggest that L3MBTL1 plays a role in promoting proliferation of transformed cells, consistent with its ability to repress p53 target gene expression.

Regulation of p21 expression by L3MBTL1 requires SET8-mediated p53K382me1. The level of total p53 protein increases with genotoxic stress, whereas SET8 and p53K382me1 levels decrease [11]; p53 occupancy on the p21 and other target promoters also increases with genotoxic stress as part of the p53-dependent induction of a DNA damage gene expression program. Based on these observations, we predicted that L3MBTL1 would disassociate from p53 upon DNA damage. To test this hypothesis, cells were treated with the radiomimetic NCS, to induce DNA double strand breaks, or vehicle control, and the interaction between endogenous L3MBTL1 and p53 was determined in co-IP assays. At basal conditions (no DNA damage), p53 was present in the L3MBTL1 IP (Fig. 3-4A). However, upon NCS treatment, which elicits a decrease in p53K382me1 levels (Fig. 3-4A), p53 was not detected in the L3MBTL1 IP. Depletion of SET8 by siRNA to emulate DNA damage-induced reduction of SET8 also abrogated the endogenous interaction between L3MBTL1 and p53 (Fig. 3-4B). Further, L3MBTL1 occupancy at the p21 promoter decreases in response to NCS treatment – in contrast to p53 binding at the p21 promoter – which as expected increases concomitant with DNA damage (Fig. 3-4C) [11]. Taken together, we conclude that the interaction between p53K382me1 and L3MBTL1 at the promoters of p53 target genes is one mechanism by which SET8-mediated methylation of p53 acts to repress p53 transactivation under basal conditions. Upon DNA damage, SET8 levels are down regulated, p53K382me1 and H4K20me1 levels are reduced, L3MBTL1 dissociates from the promoter, and transactivation is initiated by p53.
Figure 3-4

A

NCS: - +

p53
L3MBTL1
p53
p53K382me1
SET8
L3MBTL1
tubulin

L3MBTL1 IP
WCE

B

siSET8: - +

p53
L3MBTL1
p53
p53K382me1
SET8
L3MBTL1
tubulin

L3MBTL1 IP
WCE

C

L3MBTL1 occupancy at p21 promoter (% input)

0.016

0.008

0

p53 occupancy at p21 promoter (% input)

0.2

0.1

0

Control
NCS

D

DNA damage

p53

p53

p53

p53

p53

p53

SET8

L3

H4K20me1

L3

p53

p53

p53

p53

p53

SET8

L3

p53

H4K20me1

Basal Conditions

• Condensed chromatin
• Gene repression
• Open, activated chromatin
• Gene activation

• Condensed chromatin
• Gene repression
• Basal Conditions

DNA damage

p53

p53

p53

p53

p53

SET8

L3

H4K20me1

L3

p53

p53

p53

p53

p53

SET8

L3

p53

H4K20me1

Basal Conditions

• Condensed chromatin
• Gene repression
• Basal Conditions
Fig. 3-4 – The endogenous interaction between L3MBTL1 and p53 is disrupted upon DNA damage

(A) Western analysis with the indicated antibodies of L3MBTL1 IPs or WCE prepared from 293T cells, ± 0.5 ug/ml the radiomimetic NCS. Note that p53 levels do not increase upon DNA damage in 293T cells. (B) SET8 depletion abrogates the interaction between endogenous L3MBTL1 and p53. Western analysis with the indicated antibodies of the L3MBTL1 IPs or WCE prepared from 293T cells ± SET8 siRNA [11]. (C) L3MBTL1 occupancy at the p21 promoter decreases upon DNA damage. ChIP assays of endogenous L3MBTL1 at the p21 promoter in U2OS cells ± treatment with 0.5 ug/ml NCS. Occupancy values (% input) were determined by real-time PCR. (D) Model for SET8-mediated repression of p53 through promotion of p53-L3MBTL1 binding. In the absence of DNA damage, SET8 generates a population of p53 monomethylated at K382. p53K382me1 binds to and stabilizes chromatin compaction factor L3MBTL1 at the promoter regions of p53 target genes, which results in a repressive chromatin state. Upon DNA damage, K382 monomethylation levels decrease by as yet unknown mechanism (possibly via demethylation, higher methylation of p53K382, or simply is diluted out by the newly stabilized p53 protein) and this leads to L3MBTL1 dissociation from p53-target genes and a chromatin environment favorable for active gene transcription.
Discussion

In summary, we have demonstrated by biochemical, structural, and cellular approaches that L3MBTL1 functions as an effector of p53K382 monomethylation. Signaling via lysine methylation of histone proteins is a principal chromatin-regulatory mechanism that influences fundamental cellular programs[1]. Like histones, p53 is subject to a wide array of regulatory post-translational modifications that influence its biological properties [6, 8, 10]. Several lysines within the unstructured tail of p53 are methylated and the state of methylation at a specific lysine residue regulates various aspects of p53 function, including gene activation, gene repression, protein localization and protein stability [11, 32-37]. Dimethylation of p53 at K370 and K382 represent two examples of how a p53 methylation event is sensed at the molecular level; in both of these cases, the modification acts as a template for an interaction with the DNA damage response mediator 53BP1. In the context of binding to p53K370me2, 53BP1 is a co-activator of p53 transcription in response to DNA damage [36], whereas 53BP1 recognition of p53K382me2 stabilizes p53 at DNA double strand breaks and might function in communicating exposure of cells to genotoxic stress to p53 [37].

In mammals, L3MBTL1 has been linked to transcription repression. For example, L3MBTL1 interacts with the ETS transcription factor TEL to repress TEL-responsive promoters [15]. In addition, L3MBTL1 is present in a complex with RB (Retinoblastoma) protein, and functions to negatively regulate expression of a number of E2F target genes by compacting chromatin, an activity that requires MBT domain binding to repressive mono- and di- methylation marks on histones [13]. The observations that L3MBTL1 functions in part via H4K20me1 recognition and the similarity in sequence surrounding H4K20 and p53K382 led us to test binding of L3MBTL1 to p53K382me1. We propose that under basal conditions – in the absence of DNA damage – L3MBTL1 is stabilized at chromatin of p53-target genes via synergistic binding to p53K382me1 and H4K20me1 (or similar repressive mark such as H1K26me1) (see Model, Figure 3–4D). The stabilization and subsequent action of
L3MBTL1 at these p53 target genes results in chromatin compaction and thereby, L3MBTL1 – by regulating chromatin structure – indirectly renders target-DNA-bound p53 inert. In response to DNA damage, p53K382me1 levels decrease (Figure 3-4;[11]), resulting in destabilization of L3MBTL1 from p53-target genes and therefore a more favorable chromatin state, which in turn promotes transcription (Figure 3-4D).

Cabin1 binding to promoter-bound p53 was recently proposed to negatively regulate a sub-population of p53 target genes by a mechanism similar to the one proposed in our model (Figure 3-4D; [12]). These observations suggest a broader paradigm in which sequence-specific transcription factors are bound to target DNA in the absence of active transcription, but rendered quiescent by accessory factors. Such a mechanism may be important in the context of rapid activation of target genes and/or maintaining certain DNA sequences accessible.

In addition to p53, the tumor suppressor protein RB is also mono methylated by a protein lysine methyltransferase (Smyd2) – and this methylation event promotes interaction with L3MBTL1 (J. Sage, personal communications). Furthermore, the interaction between L3MBTL1 and methylated RB may play a role in repression of RB target genes (J. Sage, personal communications). Thus, L3MBTL1 might act broadly to inhibit different gene expression programs through its ability to recognize methylated non-histone nuclear proteins. Here we have demonstrated that L3MBTL1 binds to p53 in a modular manner that is dependent on SET8-mediated monomethylation at p53K382 and propose that this interaction inhibits p53 activity by regulating chromatin dynamics.

Materials and Methods

Constructs and Reagents – SET8 and p53 constructs were generated as reported previously [11]. L3MBTL1 cDNA was cloned into pCAG-Flag and pGEX6p, and pMSCVFlag Puro. L3MBTL1 mutants were generated by site directed mutagenesis (Stratagene). Antibodies used in this study are the following: αp53K382me1 [11]; horseradish peroxidase-p53 (R&D Systems); p53 (DO1) (Santa Cruz); PR-SET7 (Abcam); GST (E5) (Santa Cruz); Flag (M2) and tubulin (Sigma). Methylated p53
and histone peptides were synthesized at the W. M. Keck Facility at Yale University as previously described [11].

**Peptide Pull-down Assays** – Peptide pull-down assays were performed as previously described [22]. Briefly, 1 µg of biotinylated peptides was incubated with 1 µg of protein in binding buffer (50 mM Tris-HCl, pH 7.5, 150 - 300 mM NaCl, 0.05% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors) overnight at 4°C with rotation. After a 1-hr incubation with streptavidin beads (Amersham Biosciences) and extensive washing, bound proteins were analyzed by SDS-PAGE and Western blotting.

**Peptide Synthesis and Affinity Measurements** – The human p53 peptides were prepared by solid-phase peptide synthesis with an Applied Biosystem 431A peptide synthesizer. Peptides were assembled on Wang resin using Fmoc synthetic strategy. The coupling reaction was carried out by means of the HBTU-HOBt method. Cleavage of the peptide from the resin was achieved with TFA/water/EDT/TIS (94/2.5/2.5/1.0, v/v) for 3 hours at room temperature. After removing the resin by filtration, the filtrate was concentrated by nitrogen gas flushing, and crude peptides were precipitated by diethyl ether. Crude peptides were purified by preparative HPLC on a C18 column with water-acetonitriles system. The purity of the peptides was determined to be over 95% by analytical RP-HPLC. The mass of the peptides was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Micromass, Beverly, MA).

ITC measurements were performed using a VP-ITC calorimeter (MicroCal, Northhampton, MA). Titrations were carried out in 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 2 mM beta-mercaptoethanol at 25°C. The concentration of protein solution was estimated from the absorbance at 280 nm after the protein solutions were dialyzed. The concentration of peptide solution was determined from the base on the weight. The protein and peptide solutions were degassed before each experiment. Experiments were performed by injecting 10 µl of peptide solution (1.0 mM) into a sample cell containing 29-47 µM human L3MBTL1 (190-530). A total 29 injections were performed with a spacing 180 s and reference power of 13 µcal/s. Binding
Isotherms were plotted and analyzed using Origin software (Microcal Inc). The ITC data were fitted to a one-site binding model.

**Protein Purification and X-ray crystallography** – The human L3MBTL1 3xMBT repeats, (residues 190-530), were expressed in *E. coli* BL21(DE3) pLysS (Stratagene) grown in LB media. Bacteria were harvested by centrifugation after IPTG induction (1 mM) and lysed by sonication. The GST-fusion protein was purified on a glutathione Sepharose 4B column (Amersham), cleaved with precision protease and concentrated in Millipore concentrators (Millipore). The protein was further purified by FPLC and concentrated into 20 mM Tris pH 8.0 buffer, containing 100 mM NaCl and 5 mM dithiothreitol.

The solution of 0.25mM L3MBTL1 (residues 190-530) was incubated overnight with p53K382me1 (residues 377-386) peptide in a 1:5 molar ratio prior to crystallization. Crystals of the complex were grown using the hanging drop vapor diffusion method at 18°C by mixing 2 µl of the protein-peptide solution with 2 µl of precipitant solution containing 0.1 M sodium acetate pH 4.8, 4% PEG 4000 and 0.1 M sodium acetate. Crystals grew in a trigonal space group P3 with three molecules per asymmetric unit. Two of the three molecules, chains A and C were complexes of L3MBTL1 with the p53K382me1 peptide and the third one, chain B, was the ligand-free protein. The complete data sets were collected at 100 K on a "NOIR-1" MBC system detector at beamline 4.2.2 at the Advanced Light Source in Berkeley, CA. The data were processed with D*TREK [23]. The molecular replacement solution was generated using the program Phaser [24] and the crystal structure of L3MBTL1 (PDB 2RJE) as a search model. Crystal was detected with a twin fraction of 0.417. The initial models were built with COOT [25] and refined using the program Phenix with twin operator –h,-k,l [26]. Statistics are shown in Supplementary Table 1. The coordinates have been deposited in the Protein Data Bank under accession number (3OQ5).

**Cell Culture and Transfections** – U2OS, H1299, 293T, and HCT116 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum or newborn calf serum. Cells were transfected with plasmids or siRNA duplexes by TransIT-LT1,
TransIT-293, or DharmaFECT (Dharmacon), respectively, according to the manufacturers’ protocols.

**Coimmunoprecipitation and Western Blots** – Cotransfections for CoIP’s were performed at a 1:3 ratio Flag-L3MBTL1: SET8, with 10 ug total DNA transfected. Ectopically expressed Flag-tagged L3MBTL1 or endogenous L3MBTL1 was immunoprecipitated with Anti-Flag M2 Agarose from Mouse (Sigma) or L3MBTL1 antibody (Abcam) from WCE in cell lysis buffer (50 mM Tris-HCl [pH 7.4], 250 mM NaCl. 0.5% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM PMSF, and protease inhibitors). After incubation at 4°C overnight, the beads were washed 3x with the same buffer and boiled in 2x Sample Loading Buffer. The immunoprecipitated L3MBTL1 was resolved on SDS-PAGE gel and detected by L3MBTL1 antibody. Coimmunoprecipitated p53 was detected by HRP-αp53 to avoid crossreactivity with IgG heavy chain. p53, p53K382me1, SET8, Flag-L3MBTL1 and tubulin were detected with their respective antibodies.

**ChIP Assays** – Real-time ChIP assays were performed as previously described [11]. ChIP samples were prepared from HCT116 wt or HCT116 p53−/− stably expressing Flag-L3MBTL1 or Flag-L3MBTL1-D355N and from U2OS cells. U2OS were treated with NCS (0.5 ug/ml Sigma) for 4 hours. Occupancy values were calculated as 0.2% (M2 flag ChIP for p21) or 1% input. Primer sequences used for ChIP analysis are as follows: p21p53BS, 5’-GTGCTCTGATTGGCTTTCTG-3’/5’-CTGAAAAACAGGCAGCCCAAG-3’; p21 promoter-3, 5’-GCATGTGCTTTGTGTGAGTGT-3’/5’-GGGAGCAGGCTGTAAAAGTCA-3’; p21 coding3’, 5’-CCAGTTCATTGCACCTTGAGTAGC-3’/5’-GCCTCTACTGCCACCATTCTAAA-3’; PUMA promoter, 5’-GCGAGACTTGGGCTTTGTGT-3’/5’-CGTTCCAGGGTCCACAAAGT-3’; PUMA3’-1, 5’-GCCGAGGTGGTAGATCTCTTT-3’/5’-TCGTGTTGCCAGGAT-3’. Other primer sequences are available upon request.

**siRNA Mediated Knockdown of L3MBTL1 or SET8** – Knockdown of L3MBTL1 was performed by transfection of U2OS or H1299 cells for 48 hours with two sets of Dharmacon on-target plus siRNA duplex targeting human L3MBTL1 (5’-
GAUCUUGGUUCCUCUAAUGU-3’/5’CAUUAGAGGAACCAAGAUCU-3’ or (5’-GGUCAGUCAUAGUGGAGAAUU-3’/5’-UUCUCCACUAUGACUGACCUU-3’), respectively, by using DharmaFECT according to the manufacturers protocol. Knockdown of SET8 was performed sequentially with addition of Dharmacon on-target plus siRNA duplex targeting SET8 (5’-AGUCAAAGAUCUAUCCUAAU-3’/5’-GUACGGAGCGCCAUGAAGUUU-3’) at 0 and 24 hours for a total of 48 hours. On-target plus siControl siRNA (5’- UGGUUACUGUACAAUUA-3’) (Dharmacon) or on-target plus SMARTpool siControl siRNA were used as controls.

Stable knockdown of L3MBTL1, Growth Curves and Colony Formation Assays – Stable knockdown of L3MBTL1 was achieved using independent l3mbtl1 short hairpin (sh) RNA constructs obtained from the MISSION TRC-Hs 1.0 (Human) shRNA library (SIGMA). For growth curves, cells were counted manually every 24 hours for 7 days following initial plating on day one of 1000 cells. Standard Error of the Mean (S.E.M) was calculated at each count. For colony formation assay, 1000 cells were plated for each strain in triplicate. After two weeks media was removed and plates were washed twice with PBS, fixed and stained with 0.1% crystal violet in methanol, and rinsed with water. The mean colony number per plate was graphed for each strain. Error was calculated as S.E.M.

Reverse Transcription-PCR and Real-time PCR – Reverse transcription-PCR and real-time PCR were performed as previously described [11]. mRNA was prepared using RNeasy plus kit (QIAGEN) and reverse transcribed using First Strand Synthesis kit (Invitrogen). Quantitative real-time PCR was performed on the ABI PRISM 7700 Sequence Detection System using Taqman Gene Expression Assay primer/probe sets (Applied Biosystems). Gene expressions were calculated following normalization to GAPDH levels by the comparative cycle threshold method (Ct).

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Abbreviations used are: PKMT, protein lysine methyltransferase; MBT, malignant brain tumor; L3MBTL1, Lethal 3 Malignant Brain Tumor Like 1; ITC, isothermal calorimetry; WCE, whole cell extract; siRNA, small interfering RNA; IP, immunoprecipitation; GST, glutathione S-transferase; p53K382, p53 lysine 383; H4K20, histone H4 lysine 20; H1K26, histone H1 lysine 26, RB, retinoblastoma; me1, monomethylation; me2, dimethylation; me3, trimethylation; NCS, neocarzinostatin.
References

Chapter Four: Methylation of the retinoblastoma tumor suppressor by Smyd2*

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Summary

The retinoblastoma tumor suppressor (RB) is a central cell cycle regulator and tumor suppressor. RB cellular functions are known to be regulated by a diversity of post-translational modifications such as phosphorylation and acetylation, raising the possibility that RB may also be methylated in cells. Here we demonstrate that RB can be methylated by SMYD2 at lysine 860, a highly conserved and novel site of modification. This methylation event occurs in vitro and in cells, and it is regulated during cell cycle progression, cellular differentiation, and in response to DNA damage. Furthermore, we show that RB mono-methylation at lysine 860 provides a direct binding site for the methyl-binding domain of the transcriptional repressor L3MBTL1. These results support the idea that a code of post-translational modifications exists for RB and helps guide its functions in mammalian cells.
**Introduction**

The retinoblastoma tumor suppressor gene \( RB \) is mutated in a large spectrum of human cancers (1,2). In tumor cells where \( RB \) is not directly mutated, and in normal cells during cell cycle progression, the RB protein is functionally inactivated by phosphorylation by Cyclin/CDK complexes (3). RB phosphorylation results in the release of E2F transcription factors, allowing cells to progress into the S phase of the cell cycle (4). In addition to phosphorylation by Cyclin/CDK complexes, RB activity is controlled by other post-translational events. For instance, Chk2 phosphorylates RB in response to DNA damage (5). In addition, RB is acetylated (6-9), sumoylated (10), and ubiquitinated (11-13) in response to various cellular signals. The consequences of these modifications involve changes in RB protein levels and in the binding affinity for proteins that interact with RB, such as E2F, chromatin remodeling enzymes, and other regulators of cell cycle progression and cellular differentiation. For example, RB acetylation is thought to inhibit its phosphorylation and to promote its binding to MDM2, which results in the subsequent degradation of EID-1, an inhibitor of differentiation (8,9).

Recent evidence that non-histone proteins can be methylated supports the idea that methylation may affect gene expression and cellular functions not only by modifying histone tails (14-17) but also by changing the activity of transcription factors, including the p53 tumor suppressor (18-27). These observations suggest that, similar to the “histone code” (28), combinations of post-translational modifications may define codes that affect the function of key regulators of transcription. Based on these observations and evidence suggesting that RB directly interacts with chromatin modifying agents, including methyltransferases (29-33), it is not surprising that RB was recently shown to be a target for lysine methylation by SET9 (34). Nevertheless, the extent of RB methylation in cells and its consequences for RB function are still largely unknown. In this study, we report that SMYD2 methylates RB at lysine 860. We show that this modification permits direct binding of RB to the lysine methyl-binding protein L3MBTL1, which may alter the function of RB in cells.
Results

*SMYD2 methylates RB in vitro.* The SMYD2 methyltransferase was originally identified as an enzyme whose activity could suppress cell proliferation (35) and directly regulate p53 function (19). We tested whether SMYD2 could also methylate the RB protein, a major regulator of cell cycle progression at the G1/S phase of the cell cycle. We found that recombinant SMYD2 could methylate a C-terminal fragment of RB and had minimal or no activity against the N-terminal and large pocket fragments of the protein (Fig. 4-1A, Fig. 4-1B, left, and data not shown). We next identified lysine 860 (K860) as the residue of RB that was methylated in vitro by SMYD2 (Fig. 4-1B, right). In contrast, SET9 methylated RB within the large RB pocket and not in the C-terminal fragment containing K860 (Fig. 4-1C), suggesting a certain level of specificity for the methylation of RB by SMYD2 at K860. The conservation of RB K860 and surrounding sequences in multiple species and the fact that this region does not seem to be important in maintaining the structure of RB (44) suggest that this amino acid may have an important functional role (Fig. 4-1D).
Figure 4-1 –Methylation of human RB by SMYD2 at lysine 860 in vitro (A) Schematic representation showing RB N-terminal region (N), its pocket (A, I, B), and its C-terminal domain. Known acetylated (Ac) lysines (positions 873 and 874) and the lysine methylated by SMYD2 (position 860) are shown. (B) RB is methylated by SMYD2 at lysine 860 in vitro. Autoradiograms (³H) of histone methyltransferase (HMTase) assays with recombinant RB fragments (GST-RB) and SMYD2. Single
amino acid substitutions (K-R) are indicated. Coomassie staining shows the amount of RB protein loaded. (C) C-term RB methylation is specific to SMYD2. Autoradiogram of an HMTase assay with GST-RB fragments and SET9. (D) RB lysine 860 is highly conserved. Conservation of the sequences surrounding RB lysine 860 (in red) in several species.

To determine if RB K860 was mono-, di-, or tri-methylated by SMYD2, an RB C-terminal fragment methylated in vitro by SMYD2 was subjected to mass spectrometry analysis. While several methylated peptides were identified using this sensitive detection method, this analysis identified peptides containing mono-methylated RB K860 (Fig. 4-2), indicating that SMYD2 mono-methylates RB at K860 in vitro, similar to its activity on p53 (19).
Figure 4-2

A

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<td>K860(Me1)</td>
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<td>3+</td>
<td>K874(Me1)</td>
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<td>K896(Me1)</td>
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B

Diagram showing masses and charges with labels C3, C6, C7, Z4, Z12, Z5, Z13, [M+6H]−, [M+6H]2−.
**Figure 4-2 – Mass spectrometry analysis of RB methylation by SMYD2 in vitro**

(A) RB is mono-methylated by SMYD2. Modified peptides identified, charge state, and highest scoring site of modification (degree of methylation) in the RB/SMYD2 mass spectrometry analysis are shown. Lysine 860 is bolded and underlined. (B) Electron transfer dissociation (ETD) MS/MS spectrum matching charge state 6+ for the peptide ILVSIGESFGTSEKFQKINQMVCNSDRVLK(Me)R and corresponding fragmentation map. Matched c- and z-ions are indicated on the fragmentation map along with their m/z values. Matched c- and z-ions as well as charge-reduced precursors are labeled on the MS/MS spectrum. Lysine mono-methylation can be localized to K860 and not K844 or K847 based on site-determining ions.

*SMYD2 methylates RB in cells.* To test if SMYD2 methylates RB at K860 in cells, we developed polyclonal antibodies that specifically recognize mono-methylated K860 in the RB protein (RBK860me1). The specificity of these antibodies was verified in vitro against non-methylated, mono-, di-, and tri-methylated RB K860 and histone H4K20 peptides (Fig. 4-3A). Using these antibodies in immunoblot experiments, we were able to detect RB methylation in 293T cells that had been transfected with tagged HA-RB and MYC-SMYD2 (Fig. 4-3B). Furthermore, this methylation was dependent on the methyltransferase catalytic domain of SMYD2 because RB methylation was not detected in similar conditions where MYC-SMYD2 was substituted by MYC-SMYD2(Y290F), a catalytically inactive derivative of SMYD2 (Fig. 4-3B). Methylation was specific to lysine 860 as MYC-SMYD2 failed to methylate HA-RB(K860R), a form of RB where lysine 860 is mutated to arginine, arguing for a preferential activity of SMYD2 at K860 (Fig. 4-3B). As an additional control, we expressed a form of RB with lysine to arginine substitutions in residues close to lysine 860 (HA-RB(K870,873,874R)), two of which can be acetylated in cells (6-9). We found that these mutations did not affect the capacity of MYC-SMYD2 to methylate RB (Fig. 4-3B). Thus, RB is mono-methylated at lysine 860 by SMYD2 in cells when both proteins are overexpressed. Similarly, we found that endogenous RB molecules were methylated by MYC-SMYD2 (Fig. 4-3C). To test whether
endogenous RB methylation at lysine 860 was dependent on endogenous SMYD2 expression, we used shRNA constructs to knockdown SMYD2 in U2OS cells and found that a decrease of ~80% in SMYD2 mRNA levels resulted in a visible decrease in the amount of methylated RB that could be immunoprecipitated with the antibody against RBK860me1 (Fig. 4-3D). Similar experiments using stable knockdown in 293T cells and transient knockdown in U2OS cells produced similar results (data not shown). Together, these experiments indicate that endogenous RB is mono-methylated by SMYD2 at K860 in cells.

Figure 4-3
Figure 4-3 – Methylation of human RB by SMYD2 at lysine 860 in vivo

(A) RBK860me1 antibody recognizes mono-methyl K860 RB. The specificity of polyclonal antibodies recognizing mono-methylated K860 in the RB protein (RBK860me1) was assessed by dot blot analysis with biotinylated peptides. A peptide from histone H4 (aa 1-20) serves as a negative control. Streptavidin was used as a positive control and a loading control. (B) Overexpressed SMYD2 mono-methylates overexpressed RB at lysine 860 in 293T cells. Whole cell extracts from 293T cells transfected with the indicated plasmids were immunoprecipitated with HA resin and analyzed by immunoblot with RB, RBK860me1, and E2F antibodies. Controls include lysine to arginine substitutions in RB at K860 and K870, 873, 874 as well as a mutant form of SMYD2 (Y290F) with decreased methyltransferase activity. Inputs for HA-RB and MYC-SMYD2 are shown. (C) Overexpressed SMYD2 mono-methylates endogenous RB at lysine 860. Whole cell extracts of 293T cells were immunoprecipitated with antibodies against RB or control IgG antibodies followed by immunoblot analysis with RB and RBK860me1 antibodies. Inputs for RB and MYC-SMYD2 are shown. (D) Methylation of endogenous RB at K860 in U2OS cells is decreased upon knockdown of SMYD2. shRNA molecules against SMYD2 (shSMYD2) were stably expressed from a lentiviral vector. Cells infected with an empty lentivirus serve as a control. Whole cell extracts were immunoprecipitated with antibodies against RBK860me1 or control IgG antibodies followed by immunoblot analysis with RB. Inputs for RB and Actin are shown. The efficiency of the knockdown was measured by quantitative RT-PCR. One representative quantification of the knockdown is indicated.

RB methylation at K860 is increased in response to anti-proliferative signals.

Because RB normally inhibits the G1/S transition of the cell cycle (2), we hypothesized that RB methylation at K860 may be regulated during G1 arrest. To test this idea, we performed a cell cycle re-entry experiment. Cell cycle re-entry from quiescence upon serum stimulation was confirmed by increased RB phosphorylation,
as judged by a shift in migration, increased levels of the E2F target p107, and cell cycle profiles (Fig. 4-4A). Interestingly, the amount of RB immunoprecipitated with the antibody to RBK860me1 was higher in quiescent cells compared to cells re-entering the cell cycle while RB levels remained constant (Fig. 4-4A), indicating that RB methylation at K860 is regulated during cell cycle re-entry.

We next tested whether RB methylation at K860 is affected when cells exit the cell cycle during differentiation. To this end, we grew mouse C2C12 muscle cells under differentiation conditions. Differentiation was monitored by visual inspection of the culture and the appearance of fused myotubes and hypo-phosphorylation of RB (Fig. 4-4B). Under these conditions, RB levels and acetylated RB levels have been shown to increase transiently as cells enter the differentiation program (8). Similarly, we found a concomitant increase in total RB levels and methylated RB levels as C2C12 cells differentiated (Fig. 4-4B). This increase was not observed when RB levels were normalized (Fig. 4-4B), indicating that there is an increase in both the total pool of RB and methylated RB in C2C12 cells as they exit the cell cycle and undergo the first stages of muscle differentiation.

Finally, we tested whether RB methylation by SMYD2 was affected by DNA damage, as was shown for p53 (19). We treated NIH 3T3 cells with the DNA damaging agent etoposide and verified the induction of DNA damage by immunoblot analysis with antibodies to the phosphorylated form of histone H2AX (γH2AX) (Fig. 4-4C). We found that the amount of RB that could be immunoprecipitated with the antibody to RBK860me1 increased after etoposide treatment while RB levels remained constant, indicating that RB methylation at K860 is induced by DNA damage (Fig. 4-4C). Thus, RB methylation at K860 is dynamic and regulated during several cellular processes, suggesting that it may play a functional role in mammalian cells.
Figure 4-4 – Regulation of RB methylation at lysine 860 in vivo

(A) Methylation of RB accumulates in G0. Quiescent T98G cells were stimulated with 20% serum and RB methylation was measured by immunoprecipitation of whole cell extracts with RBK860me1 antibody and immunoblot analysis with RB antibody. Note that RB becomes hyperphosphorylated as cells re-enter the cell cycle, and that the levels of the RB/E2F target p107 increase as cells progress into S phase. Actin serves as a loading control. Propidium iodide FACS profiles (bottom) show cell cycle re-entry. (B) Methylated RB accumulates as C2C12 myoblasts differentiate. Whole cell extracts from differentiating C2C12 myoblasts were immunoprecipitated with
RBK860me1 antibody followed by immunoblot for RB. Input levels of RB are shown along with Actin as a loading control (top). A replicate of this experiment is shown with RB loading equalized from day 0 to day 2 (middle). Photographs at days 0 and 2 show the differentiation into myotubes (bottom). (C) RB methylation accumulates with DNA damage. Whole cell extracts from NIH3T3 cells treated with etoposide for the indicated time periods were immunoprecipitated with RBK860me1 followed by immunoblot analysis. Immunoblotting input with the phospho-H2AX epitope (γH2AX) serves as a positive control for the accumulation of DNA damage. Input levels of RB are shown along with Actin as a loading control.

RB methylation at K860 regulates RB binding to the transcriptional repressor L3MBTL1. The observation that the amount of methylated RB increases under cytostatic conditions, and the fact that co-expression of SMYD2 and RB in RB mutant Saos-2 cells causes further repression of E2F target genes when compared with RB alone (data not shown), raised the question of how methylation of RB might affect its function. One possibility was that RB methylation may change its binding to specific cellular partners. While E2F1 binds to the C-terminus of RB (6), K860 contributes a weak interaction with E2F1 (44), and we found no reproducible differences in the RB/E2F1 interaction with or without SMYD2, in cells and in vitro (Fig. 4-3C and data not shown). This observation did not exclude that the binding to other partners may be affected by the methylation event.

L3MBTL1 is a histone methyl-binding protein that can condense chromatin and repress gene expression, including the expression of RB targets (45,46). The MBT domain of L3MBTL1 has been found to bind methyl groups, with a preference for mono- and di-methylated lysines (45,47,48). While there is no published evidence that RB and L3MBTL1 directly interact, we hypothesized that RB methylation may serve as a signal to increase a potential interaction between RB and L3MBTL1. We found that a recombinant 3xMBT domain from L3MBTL1 bound more efficiently to RB peptides mono- and di-methylated at K860 than it did to non- and tri-methylated peptides (Fig. 4-5A). In addition, mutations in amino acids of L3MBTL1 predicted to
alter its ability to recognize methylated peptides (46,49,50) decreased its ability to bind to mono-methylated RB at K860 (Fig. 4-5B). Isothermal titration calorimetry assays further confirmed the specificity of L3MBTL1 for mono- and di-methylated RB K860 (Figs. 4-5C and 4-5D), with an affinity slightly higher than what was described with mono- and di-methylated histone peptides (46).

**Figure 4-5**
Figure 4-5 – RB methylation at K860 increases its interaction with the MBT domains of L3MBTL1 in vitro

(A) 3xMBT from L3MBTL1 preferentially binds mono- and di- methyl K860 RB fragments. Pull-down assays of biotinylated RB and H4 peptides containing varying degrees of methylation with GST tagged 3xMBT from L3MBTL1. (B) Mutations in key residues of the methyl-binding pocket of 3xMBT L3MBTL1 reduce binding to methylated RB fragments. Pull-down assays of biotinylated RBK860me0 and RBK860me1 peptides with 3xMBT L3MBTL1 mutants. Input levels of the RB fragment are shown to the left of each blot. (C, D) Calorimetry assays confirm and quantify binding of methylated RB to 3xMBT L3MBTL1. Isothermal titration calorimetry assays were performed to measure the affinity of the interaction between RB and the 3xMBT domain of L3MBTL1. Only the interaction with the mono-methylated RB peptide is shown in C. All the data are quantified in D; no Kd could be determined for K860me0 and K860me3 due to the low affinity of the interaction.

To determine if methylated RB associates with L3MBTL1 in cells, we first determined if we could detect methylated RB in chromatin where L3MBTL1 has been shown to function as a repressor (45). Indeed, the analysis of different cellular fractions from 293T cells transfected with HA-RB and MYC-SMYD2 revealed mono-methyl RB at K860 in the cytoplasmic (S2), nuclear (S3), and most importantly, chromatin (Chr) fractions (Figure 4-6A). Next we investigated the binding of methylated RB to L3MBTL1 in cells through co-immunoprecipitation experiments. We found that FLAG-L3MBTL1 co-immunoprecipitated more HA-RB in the presence of MYC-SMYD2 (Figure 4-6B). Furthermore, similar to the observations with recombinant peptides (Fig. 4-5), the interaction between RB and L3MBTL1 was dependent on an intact 3xMBT domain (Fig. 4-6C). Finally, this interaction was decreased in cells expressing MYC-SMYD2 and HA-RB(K860R) compared to cells expressing wild-type HA-RB, indicating that methylation at lysine 860 was required for the optimal binding of L3MBTL1 to RB (Fig. 4-6D).
Figure 4-6 – RB methylation at K860 increases its interaction with the MBT domains of L3MBTL1 in vivo

(A) Mono-methyl K860 RB can be localized to chromatin. 293T cells were fractionated by detergent lysis to generate a cytoplasmic S2 fraction, a nuclear S3 fraction, and a chromatin (Chr) fraction. Fractions were then analyzed by immunoblot with antibodies against RB and RBK860me1. Tubulin and H3K4tri-me were used to assess the purity of the fractionation. (B) Overexpression of SMYD2 increases binding of RB to L3MBTL1. 293T cells were transfected with the indicated expression plasmids. Whole cell extracts were immunoprecipitated with FLAG resin and analyzed by immunoblot with antibodies against RB, RBK860me1, and L3MBTL1. Inputs for FLAG-L3MBTL1, MYC-SMYD2, and RB are shown. (C) The D355N mutation in the methyl-binding pocket of L3MBTL1 reduces the affinity of L3MBTL1 for RB. 293T cells were transfected with the indicated expression plasmids. IP was performed as in B and analyzed by immunoblot with antibodies against RB, RBK860me1, and L3MBTL1. Inputs for FLAG-L3MBTL1, FLAG-L3MBTL1(D355N), RB, and MYC-SMYD2 are shown. (D) RB(K860R) mutation reduces the affinity of RB for L3MBTL1. 293T cells were transfected with the indicated expression plasmids. Immunoprecipitation was performed as in B and C and analyzed by immunoblot with antibodies against HA, RBK860me1, and L3MBTL1. Inputs for FLAG-L3MBTL1, HA-RB, HA-860, and MYC-SMYD2 are shown. Tubulin serves as a loading control.
Discussion

Regulation of RB activity in cells is thought to be largely, although not entirely (51), due to post-translational modifications. Here we describe a previously unidentified post-translational modification of RB, methylation of K860 by SMYD2. We also show that RB K860 methylation facilitates a direct interaction between RB and the methyl-binding protein L3MBTL1. The identification of this modification provides further evidence for the existence of an “RB code” whereby specific patterns of modifications affect one another and dictate the myriad of RB cellular functions.

Our data indicate that SMYD2 methylates RB at K860 in vitro and in cells but does not exclude the possibility that SMYD2 may methylate RB at different sites under specific conditions. It is also possible that SMYD2, or another methyltransferase, may generate K860me2 and/or K860me3 residues. Furthermore, other methyltransferase activities could control the functions of RB in distinct cellular contexts, an idea supported by our finding that SET9 can also methylate RB in vitro, albeit at a different site than SMYD2. Future experiments will continue to examine the spectrum of RB methylation in relation to cellular context and other post-translational modifications.

Early studies of SMYD2 described its ability to inhibit cell cycle progression (35). The identification of RB as a SMYD2 substrate, and the accumulation of methylated RB molecules during conditions where RB represses cell cycle genes, may provide a mechanism for this effect, perhaps in conjunction with the effects of SMYD2 on p53 (19). Nevertheless, additional experiments will be required to test this model. In particular, the genetic interactions between SMYD2, RB, and p53 during embryonic development at a time when many cells exit the cell cycle remain to be tested. In addition, a clear role for SMYD2 in tumorigenesis has yet to be identified.

L3MBTL1 is a mono- and di-methyl histone binding protein that has the ability to condense chromatin and repress transcription (52). A possible biochemical link between L3MBTL1 and RB has been suggested by the fact that both proteins have been found in the same large complexes in flies and human cells (45,53). In
addition, L3MBTL1 has the ability to bind to and repress the transcription of some E2F target genes (45). However, these studies did not determine if RB and L3MBTL1 directly interacted. Our studies indicate that RB can bind directly to the 3xMBT domain of L3MBTL1. Moreover, this direct interaction is facilitated by RB methylation at K860. These associations between RB and L3MBTL1 suggest a model whereby RB methylation by SMYD2 may serve to recruit L3MBTL1 to the promoters of specific RB/E2F target genes to repress their transcription. Similar observations were recently made with p53, which indicate that the L3MBTL1/p53 interaction serves to control the repression of p53 target genes in the absence of cellular stress (Or Gozani, personal communication). Since L3MBTL1 has been shown to homodimerize (54), future studies will determine if L3MBTL1 bound to methylated RB (and p53) also has the potential to bind methylated histones on the same monomer or as a dimer. This would provide a direct physical link between the “RB code”, the “histone code”, and structurally repressed chromatin. Because L3MBTL1 is the homolog of the Drosophila tumor suppressor protein dL(3)MBT (52), future experiments should also investigate the tumor suppressor function of L3MBTL1 in mammals.

Materials and Methods

Plasmids, Antibodies, and Peptides. MYC-SMYD2, MYC-SMYD2(Y290F) (35), FLAG-SMYD2 (19), GST-SET9 (18) were described previously. Details for the construction of human GST-RB fragments, HA-RB(K860R), HA-RB(K870,873,874R), GST-SMYD2, GST-3xMBT, GST-3xMBT mutants, and FLAG-L3MBTL1 wild-type and mutant vectors are available upon request. Biotinylated RB peptides VCNSDRVLK(me0-3) RSEAGSNPPKPLKKLK and H4K20(aa 1-23) (me0-3) peptides were purchased from the Small Scale Peptide Synthesis facility (Yale University). Rabbit polyclonal antibodies to RBK860me1 were generated by Abmart following immunization with the mono-methylated peptide 853-CNSDRVLK(me1) RSEAG-865. Other antibodies used in this study are directed against Streptavidin (Abcam), RB ((36) - 4.1, DSHB, University of Iowa), RB C-15
(Santa Cruz Biotechnology), E2F1 (Santa Cruz Biotechnology), MYC (Sigma Aldrich), β-Actin (Sigma Aldrich), p107 (Santa Cruz Biotechnology), γH2AX (Cell Signaling Technology), GST (E5, Santa Cruz Biotechnology), FLAG (M2, Sigma Aldrich), α-Tubulin (Sigma Aldrich), L3MBTL1 (Abcam), and H3K4trime (Active Motif).

**Cell Culture and Transfections.** 293T, T98G, Saos-2, U2OS, C2C12, and NIH3T3 cells were maintained in DMEM medium supplemented with 10% bovine growth serum. Cells were transfected with calcium phosphate or using a Nucleofector (Lonza).

**Immunoprecipitation.** Whole cell extracts from cells were prepared in 50 mM Tris pH 7.4, 250 mM NaCl, 10% glycerol, 0.5% Triton-X-100, and protease inhibitors. RB was immunoprecipitated with RB or RBK860me1 antibodies. Ectopically expressed HA-RB and mutants were precipitated with anti-HA agarose beads (Sigma Aldrich) and ectopically expressed FLAG-L3MBTL1 and mutants were immunoprecipitated with anti-M2 FLAG resin (Sigma Aldrich). Immunoprecipitated proteins were resolved on SDS-PAGE gels for immunoblot analysis as described (36).

**In Vitro Methyltransferase Assay and Mass Spectrometry.** Assays were performed as previously described (37). Briefly, 5 µg of GST-RB were incubated with 5 µg of GST-SMYD2 or GST-SET9, and 0.1 mM S-adenosyl-methionine (SAM, Sigma Aldrich) or 55 µCi ³H-SAM (Perkin Elmer) at 30°C for 2 hours before electrophoresis and autoradiography or mass spectrometry.

**Mass spectrometry analysis.** Samples were first reduced and alkylated with 10 mM Tris(2-carboxyethyl) phosphine hydrochloride (Roche Applied Science) and 55 mM iodoacetamide (Sigma Aldrich), respectively, then digested with endoproteinase Arg-C (Roche Applied Science) according to the manufacturer’s specifications. The protein digest was pressure-loaded onto a 100 µm i.d. fused silica capillary (Polymicro Technologies) analytical column with a 5 µm pulled-tip, packed with 10 cm of 5 µm C18 resin (Phenomenex). The analytical column was placed inline with an 1100 quaternary HPLC pump (Agilent Technologies) and the eluted peptides were electrosprayed directly into an LTQ-XL mass spectrometer (Thermo Scientific). The
buffer solutions used were 5% acetonitrile/0.1% formic acid (buffer A) and 80% acetonitrile/0.1% formic acid (buffer B). The 120 min elution gradient had the following profile: 10% buffer B at 5 min, to 50% buffer B at 80 min, to 100% buffer B from 90-100 min. A cycle consisted of one full scan mass spectrum (400-2000 m/z) followed by 5 data-dependent electron transfer dissociation (ETD) MS/MS spectra with an isolation width of 2 m/z. Fluoranthene anions were set to a target value of $10^5$ and ETD reaction time was set at 50 ms. Supplemental activation was enabled. Application of mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Scientific). ETD MS/MS spectra were extracted up to charge state 10+ using RawXtract (version 1.9) (38). ETD MS/MS spectra were searched with the ProLuCID algorithm (39) against the *E. coli* SGD database supplemented with the GST-RB (aa 829-928) fusion protein sequence and concatenated to a decoy database in which the sequence for each entry in the original database was reversed (40). Charged reduced precursors were removed from the spectra prior to searching. The ProLuCID search was performed using full enzyme specificity and a static modification of cysteine due to carboxyamidomethylation (57.02146). Differential modifications considered were lysine mono-methylation (14.0157), di-methylation (28.0314), and tri-methylation (42.0471). ProLuCID search results were assembled and filtered using the DTASelect2.0 algorithm (41) with a false positive rate below one percent.

**shRNA-Mediated Knockdown.** A pGIPZ lentiviral vector containing shRNA against *SMYD2* (Open Biosytems) or a control vector were used to infect cells as described (42). For transient knockdown, U2OS were transfected with two rounds of *SMYD2* ON-TARGETplus SMARTpool (L-020291-00-0005) or ON-TARGETplus Non-targeting siRNA#1 (D-001810-01-05) using DharmaFECT 1 (Thermo Scientific).

**RT-PCR and Real-Time PCR.** RNA was extracted with TRIzol (Invitrogen) and the RNeasy Mini Kit (QIAGEN). RT-PCR and quantitative Real-Time PCR were performed using the DyNAmo cDNA synthesis kit and the SybrGreenER Mastermix (Invitrogen), respectively. Primer sequences are available upon request.

**Cell Cycle and Cell Death and Differentiation Assays.** T98G cells were serum starved...
in DMEM supplemented with 0.1% bovine growth serum and then stimulated with DMEM supplemented with 20% bovine growth serum for 0, 9, and 18 hrs. For flow cytometry, T98G cells were collected and fixed with 70% ethanol at 4°C. Cells were then washed with PBS and stained with 10 µg/ml propidium iodide and 100 µg/ml RNase. DNA damage assays were performed in NIH3T3 cells treated with 10 µM etoposide. C2C12 myoblasts were induced to differentiate by growing cells to confluency in medium with 2% horse serum for 0, 1, 2, and 7 days.

**Peptide pull-down assays:** Peptide pull-down assays were performed as previously described (37). Briefly, 1 µg of biotinylated peptide was incubated with 1 µg of protein in binding buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% NP-40, and protease inhibitors) overnight at 4 °C with rotation before incubation with streptavidin beads (Amersham Biosciences) and immunoblot analysis.

**Isothermal titration calorimetry.** ITC assays were conducted with a MicroCal VPITC Calorimeter as recently described (43). Data were analyzed with the Origin calorimetry software package assuming a one site-binding model. N-values, reflecting the stoichiometry of the RB-3xMBT complex, were between 0.8 and 1.2. Experiments were repeated 2-4 times, and the reported error is the standard deviation of each set of measurements.

**Cell Fractionation.** 2x10^7 cells were incubated in Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M Sucrose, 10 % Glycerol, 1 mM DTT, 0.1% Triton-X-100, 5µg/ml leupeptin, 5µg/ml aprotinin, 0.1 mM PMSF) for 10 min on ice. Nuclei were collected by centrifugation at 1,300 g at 4°C for 5 min and the supernatant (S1) was further clarified by centrifugation at 16,000 g at 4°C for 10 min to yield a cytoplasmic fraction (S2). The nuclei were washed once in Buffer A and then lysed in Buffer B (3mM EDTA, 0.2 mM EGTA, 1 mM DTT, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 0.1 mM PMSF) for 15 min on ice followed by centrifugation at 1,700 g at 4°C for 5 min. The supernatant (S3) consisting of nucleoplasm was removed and the pellet (P3) was resuspended in Laemmli buffer and boiled for 20 min to yield the chromatin fraction (Chr).
**Statistical significance.** Student t-tests were conducted in order to determine statistical significance. * denotes p value < 0.05.

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The abbreviations used are RB, retinoblastoma protein; SMYD2, SET- and MYND-containing protein 2; MBT, Malignant Brain Tumor; CDK, Cyclin-Dependent Kinase; GST, Glutathione-S-transferase.
References


Chapter Five: Discussion
The focus of this thesis has been to characterize the molecular mechanisms through which PKMTs mediate non-histone protein lysine methylation in order to regulate the function of multifunctional tumor suppressor proteins such as p53 and RB. The mechanisms first characterized on histones such as effects on stability, crosstalk between modifications, or interaction with effector proteins, are readily reproduced on p53. Furthermore a PKMT may often coordinate concurrent action on histone and non-histone substrates leading to an integrated downstream outcome such as chromatin condensation and gene silencing, or chromatin relaxation and gene activation. SET8 represents one such example of synchronized methylation activity: under normal conditions, during S-phase, SET8 is expressed and tracks with the replication fork placing H4K20 mono- and trimethylation marks essential to genome replication and stability (1). SET8 contemporaneously monomethylates p53 to increase concentrations of the inactive p53K382me1 species, thus maintaining p53 quiescent (though poised) at a time when its tumor suppressing activities would derail healthy cell proliferation (2,3). In this way p53 regulation by lysine methylation represents a logical extension of the post translational modification code already characterized on histones, in a fashion that frequently supports coordinated response to various intracellular circumstances.

Ultimately, no lysine methylation event is essential to the overall tumor suppressing activity of p53. This point was succinctly recapitulated in mouse genetic studies demonstrating that p53K6R and p53K7R mutants (all C-terminal domain lysines mutated to arginine) are functionally equivalent to wildtype p53 with respect to stability, transactivation, and induction of apoptosis (4,5). Rather lysine methylation delineates the finer points of p53 sensitization in response to diverse stimuli by generating specific populations that intercommunicate and transition through crosstalk to either augment or inhibit p53 activity via interaction with diverse effector proteins. These events contribute to the overall sensitivity and robustness of a p53 “metastable” equilibrium that promotes homeostasis under normal conditions but achieves high
level specificity in a gene specific, promoter specific fashion upon genotoxic stress (6).

What are the broader implications of lysine methylation as a regulatory mechanism of non-histone proteins? One interesting observation made in the Huang et al study on G9a and Glp methylation of p53 is that numerous cancers overexpress either G9a or Glp. If fifty percent of cancers exhibit mutations in the p53 gene, then of course the other 50% possess wild type p53, at least at the gene level. Overall, the negative regulation of p53 function by lysine methyltransferases such as G9a/Glp, Smyd2, and SET8, or other effector proteins such as L3MBTL1, indicates that aberrant activity of these proteins may allow them to function in an oncogenic capacity through their restraint of wild type p53 function. If this is the case, then this group of negative regulators of p53 may represent a novel class of therapeutic targets particularly for those cancers with an unmutated p53 gene.

Apart from p53, numerous non-histone proteins are methylated on lysine residues (7-9). For the most part the mechanisms and biological implications of these events are not wholly understood. However, several mechanistic themes identified in PTM regulation of p53 have been recently been extended to other transcription factors such as RB and RelA (10-12). These studies suggest that regulation by lysine methylation represents a broader paradigm for fine-tuned modulation of non-histone protein activity.
References

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