

## Post-translational control of transcription factors: Methylation RankS highly

Simon M. Carr\*, A. Poppy Roworth\*, Cheryl Chan and Nicholas B La Thangue<sup>1</sup>

\* these authors contributed equally to the work

1. To whom correspondence should be addressed. E-mail: [nick.lathangue@oncology.ox.ac.uk](mailto:nick.lathangue@oncology.ox.ac.uk),  
Tel: +44 1865 617091, Fax: +44 1865 617092

Laboratory of Cancer Biology,

Department of Oncology,

University of Oxford,

Old Road Campus Research Building,

Old Road Campus

off Roosevelt Drive,

Headington, Oxford, OX3 7DQ

United Kingdom

**Running Title:** Methylation in transcription factor control

## **Keywords**

pRB, E2F1, p53, NFκB, arginine methylation, lysine methylation, PRMT, PKMT, transcription

## **Abbreviations**

53BP1: p53 binding protein 1; ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; APAF: apoptotic protease activating factor; ARF: ADP-ribosylation factor; ATM: ataxia telangiectasia mutated; ATR: ataxia telangiectasia and RAD3-related; BCL: B-cell lymphoma family protein; BCR: B-cell receptor; BIM: BCL2-like protein 11; BMP: bone morphogenetic proteins; CDC: cell division cycle protein; CDK: cyclin dependent kinase; CHK: checkpoint kinase; DHFR: dihydrofolate reductase; DOT1L: Disruptor of telomeric silencing 1-like; E2F1: E2F transcription factor 1; EHMT: euchromatic histone-lysine N-methyltransferase; ELMO: engulfment and cell motility family protein; EZH2: Enhancer of zeste homolog 2; FBXL: F-box and leucine-rich repeat protein; GADD45: growth arrest and DNA-damage-inducible protein; HP1: heterochromatin protein; IκB: inhibitor of kappa B; IGF: insulin-like growth factor; IL: interleukin; JMJD: jumonji-domain containing protein; KDM: lysine demethylase; L3MBTL1: L(3)MBT-like 1 protein; LSD: lysine specific demethylase; MBT: malignant brain tumour domain; MCP: monocyte chemoattractant protein-1; MDM2: mouse double minute protein; Me: Methyl; Me2a: asymmetric dimethyl; Me2s: symmetric dimethyl; MEF: mouse embryonic fibroblast; MLL: myeloid/lymphoid or mixed-lineage leukemia; NFκB: nuclear factor kappa B; NHEJ: non-homologous end joining; NSD: nuclear receptor binding SET domain protein; p53: tumour protein 53; P53AIP: p53-regulated apoptosis-inducing protein 1; p73: tumour protein 73; P/CAF: p300/CBP associated factor; pRB: retinoblastoma protein; PAD: protein arginine deiminase; PHD: plant homeo-domain containing protein; PHF: PHD-type zinc finger; PP2A: Protein phosphatase 2A; PKMT: protein lysine methyltransferase; PRMT: protein arginine methyltransferase; PUMA: p53 upregulated modulator of apoptosis; PWWD: Pro-trp-trp-aspartic domain; RHD: REL-homology domain; RASSF1A: ras -association domain family 1 isoform A; SET: su(var)3-9, Enhancer-of-zeste and Trithorax domain containing protein; siRNA: small interfering RNA; SMYD2: SET and MYND domain containing protein 2; TCR: T-cell receptor; TIP60: TAT-interactive protein; TK: thymidine kinase; TLR: toll-like receptor; TNF: tumour necrosis factor; UTX: Ubiquitously transcribed X chromosome tetratricopeptide repeat protein; WD40: Trp-Asp repeat domain; γH2AX: histone H2A member X phosphorylation

## **Abstract**

Methylation of lysine and arginine residues on histones has long been known to determine both chromatin structure and gene expression. In recent years, the methylation of non-histone proteins has emerged as a prevalent modification which impacts on diverse processes such as cell cycle control, DNA repair, senescence, differentiation, apoptosis, and tumourigenesis. Many of these non-histone targets represent transcription factors, cell signalling molecules and tumour suppressor proteins. Evidence now suggests that the dysregulation of methyltransferases, demethylases, and reader proteins is involved in the development of many diseases, including cancer, and several of these proteins now represent potential therapeutic targets for small molecule compounds, fuelling a recent surge in chemical inhibitor design. Such molecules will greatly help us to understand the role of methylation in both health and disease.

## Introduction

Post-translational modifications of histone proteins have long been known to play a critical role in defining chromatin structure and regulating gene expression [1, 2]. Among these various modifications, methylation of lysine and arginine residues are particularly important in regulating gene transcription programs [1]. These modifications are mediated by a group of enzymes known as the protein methyltransferases, which are composed of two families: the protein lysine methyltransferases (PKMTs) and the protein arginine methyltransferases (PRMTs). The  $\epsilon$ -amino group of lysine may be mono-, di-, or trimethylated by the PKMTs in an S-adenosylmethionine (SAM)-dependent manner, whilst arginine may be mono- or dimethylated by the PRMTs (Figure 1). Dimethylation of arginine can occur in an asymmetric (Me2a) or symmetric (Me2s) fashion, determined by the addition of the two methyl groups to a single guanidino nitrogen atom, or two nitrogen atoms respectively (Figure 1). The orientation of methyl groups on arginine residues is determined by the type of PRMT involved: type I PRMTs (PRMTs 1, 3, 4, 6, 8) catalyse Me2a, whilst type II PRMTs (PRMTs 5 and 9) catalyse Me2s. A third class of enzymes, known as type III PRMTs, are only able to mono-methylate their substrates (PRMT 7). Although some of the PKMTs and PRMTs can be considered histone specific, many have now been found to modify both histone and non-histone substrates [3-5]. Methylation of non-histone proteins appears to be an important post-translational modification with wide-ranging cellular functions, impacting on diverse processes such as cell signal transduction and tumourigenesis [3-5]. Tables 1 and 2 contain a brief list of known arginine and lysine methylation sites on transcription factors, although these are not meant to be exhaustive.

Protein methylation was initially believed to be irreversible in nature, due to the observation that the half-life of histone methylation was almost equivalent to the half-life of the histones themselves. However, in the last decade a number of lysine specific demethylases have been identified, including the amine oxidases and the jumonji C (JmjC)-domain-containing enzymes [6, 7], indicating that protein lysine methylation is dynamically regulated. Intriguingly however, a bona fide arginine demethylase has yet to be identified, though some studies suggest that JMJD6 could act in this fashion [8]. Arginine residues can also be covalently modified by protein arginine deiminases (PADs) to produce citrulline, and this modification will thus antagonise arginine methylation [6]. However, whilst methyl-arginine can also act as a substrate for the PADs *in vitro*, these enzymes work much less efficiently on methylated arginine substrates than on non-methylated ones, causing speculation on the relevance of demethylination as a method for demethylating arginine in cells [6].

Increasingly, it is becoming apparent that the amine oxidases and jumonji demethylase enzymes can target non-histone proteins in addition to methylation sites in histones, and these dynamic changes in protein methylation can have important implications for the regulation of apoptosis, DNA repair, ribosomal function, and cell cycle regulation [7].

Similar to other forms of post-translational modification, methylation can be important for mediating protein-protein interactions by acting as a docking platform to recruit 'reader' proteins that contain domains capable of recognising methyl-lysine and methyl-arginine. To date, readers of methyl-lysine are the most thoroughly characterised group and include chromodomain, MBT, PHD and Tudor domain containing proteins [9]. Readers for methyl-arginine are less well described, with Tudor-domain containing proteins being the most investigated to date [10]. In all cases, recruitment of reader proteins to methyl marks often constitutes an integral part of cell signalling events, which acts to connect the generation of protein modifications with their downstream physiological response.

In this review, we will summarise recent findings on non-histone protein methylation, with particular focus on a number of transcription factors implicated in cell cycle control and inflammation. For each transcription factor, we will highlight the evidence for both lysine and arginine methylation and discuss how these modifications impact on protein function. We will also examine the importance of reader proteins in determining a cellular response to methylation, and the enzymes responsible for removing these methyl marks where they are known.

## **E2F-1**

The E2F family of transcription factors help control cell cycle progression by regulating a set of genes, such as CYCLIN D, CDK4 and CDC25, which are required for G1/S transition and DNA replication. These transcription factors can also positively regulate apoptosis in response to DNA damage via upregulation of pro-apoptotic target genes such as BIM, APAF1 and p73 (for a recent review see [11]). However, it has been suggested that E2F-1 may play the largest role in regulating E2F-dependent apoptosis [12], and this family member is the one we will focus on in this review. With such diverse roles, much interest has been placed on trying to identify switches which drive these differing processes and ultimately cell fate.

In an elegant study, arginine methylation of E2F-1 was shown to occur at three sites: R109, R111 and R113 (Figure 2A) [13, 14]. This is of particular interest as these arginine residues lie within an RGRGR motif, which is not present in the other E2F proteins, suggesting that this is a specific modification of E2F-1. These methylation marks (either R109Me2a or R111/113Me2s) generate opposing cell fate decisions by E2F-1 and appear to show some cross talk, as methylation at R109 inhibits methylation at R111/113 and vice versa [14]. R109 is asymmetrically dimethylated by PRMT1 after DNA damage and was shown to result in an increased E2F-1 presence on the promoters of apoptotic target genes such as p73 and APAF1. This correlated with an increase in p73 and Apaf1 protein levels and a subsequent increase in apoptosis. Accordingly, an R109K mutant was able to confer resistance to apoptosis following DNA damage and showed reduced recruitment to pro-apoptotic target gene promoters under these conditions. Conversely, symmetric methylation of both R111 and R113 by PRMT5 was shown to be associated with cell cycle progression: Cyclin A binding to E2F-1 was shown to inhibit PRMT1-mediated methylation, instead favouring an R111/113Me2s state which corresponded to E2F-1 presence on growth promoting target genes such as TK, CYCLIN A and CDC6 [13, 14]. Loss of these sites in a double mutant (R111/113K) resulted in much slower cell growth and proliferation. Importantly, a reader of the R111/113Me2s mark was also identified – the tudor domain containing protein p100-TSN [14]. This protein is involved in RNA processing and transcriptional regulation and was shown to increase the stability of E2F-1 and contribute to reducing apoptotic activity after symmetric methylation [15, 16]. In line with these results, a separate study has identified PRMT5 over-expression in ovarian cancer – both in clinical samples and from a mouse model of the disease – and depletion of PRMT5 in ovarian cancer cell lines was shown to inhibit growth and induce apoptosis in an E2F-1-dependent manner [17].

E2F-1 has also been discovered to be monomethylated at K185 by SET7/9, and subsequently demethylated by LSD1 [18, 19]. However, the two reports that describe this event report contrasting effects of lysine methylation on E2F-1. In the earlier paper, Kontaki and Talianidis [18] showed that K185 methylation occurred after DNA damage and lead to E2F-1 destabilisation as well as inhibiting other post-translational modifications, such as CHK1 mediated phosphorylation and P/CAF mediated acetylation. Ultimately, they suggested that this would protect the cell from apoptosis as reduced levels of E2F-1 will limit the expression of pro-apoptotic target genes after DNA damage. Therefore, subsequent demethylation by LSD1 would result in accumulation of E2F-1 (due to increased protein stability) which can then be phosphorylated at S364 (CHK1) and acetylated at K117, K120 and K125

(P/CAF) resulting in apoptotic target gene (APAF1/p73) transcription [18]. In contrast, the report by Xie *et al.* [19] suggested that SET7/9 methylation of E2F-1 after DNA damage stabilised the protein by reducing its proteasomal degradation as well as decreasing its overall DNA binding ability. Additionally, this paper reported that E2F-1 methylated at K185 was recruited to pro-apoptotic target gene promoters such as BIM, where it consequently induced apoptosis following DNA damage [19]. A further report identified K185Me as a requirement for subsequent NEDDylation of E2F-1, NEDD-dependent protein turnover and reduced cellular growth rates [20]. This would lend support to the view that the K185Me mark is destabilising, as described by Kontaki and Talianidis. However, NEDDylation has also been shown to reduce E2F-1 binding at pro-apoptotic but not pro-growth target gene promoters [21], which would suggest that NEDDylated (and K185 methylated) E2F-1 could be inhibiting apoptosis. After DNA damage, NEDDylation of E2F-1 will decline, permitting the induction of E2F-dependent apoptotic genes. A further study showed that inhibiting methylation of K185 resulted in increased expression of the RASSF1A tumour suppressor, and corresponded with an increase in apoptosis [22]. However, the compounds used in this paper were not selective, acting to reduce the pool of available methionine. This would therefore impact all methylation events in cells (DNA, protein, lipids), so the effect on E2F-1 and its activity should be considered carefully in this context [22]. Clearly, the function of K185Me requires more study and future work in this area should pin-point the precise biology of lysine-methylated E2F-1. It will also be important to elucidate the interplay between methylation and other modifications, such as NEDDylation, to ultimately understand the E2F-dependent cellular outcome.

## **pRB**

The retinoblastoma protein family members (pRB, p107, p130) function to inhibit E2F-dependent transcription, and so represent one of the key cell cycle regulators that govern the transition from G1 to S phase [11]. Of the family of 'pocket proteins', pRB is by far the most highly studied. It can suppress the activity of the E2F family by directly binding to their transactivation domains, and by recruiting co-repressors and chromatin remodelling factors to E2F-responsive gene promoters [23, 24]. The importance of pRB in cell cycle control is highlighted by the fact that the pRB pathway is mutated in almost all human cancers, either by direct mutation of the gene itself, or by dysregulation of pRB's many upstream regulators [25]. However, like E2F-1, pRB functions in several other processes beyond the G1-S phase transition, including differentiation, apoptosis, senescence and the DNA damage response [26].

To date, only a single arginine methylation event has been described for pRB, though it seems likely that others will be identified in time. PRMT4, an enzyme whose overexpression has been implicated in many forms of cancer, can target RMe2a of several arginine residues in the C-terminus of pRB. Although mass spectrometry confirmed methylation at R775, R787 and R798, only R787 could be detected in cells using a site-specific antibody (Figure 2B) [27]. Arginine methylation appeared to impact on pRB phosphorylation at some neighbouring CDK sites as mutation of all three arginine residues in pRB caused a reduction in phosphorylation at S788, S795 and T821, but not S807 or S811. This suggests that PRMT4-mediated arginine methylation of pRB can promote cell cycle progression by enhancing phosphorylation of CDK sites which causes the release of E2F-1 and drives transcription of E2F-responsive genes. Accordingly, depletion of PRMT4 by small interfering RNA (siRNA) resulted in an increased interaction between pRB and E2F-1, and the triple R to K mutant repressed E2F-1-dependent transcription more efficiently than wild type pRB in a reporter assay [27]. PRMT4-targeted pRB arginine methylation may therefore provide another possible mechanism for the oncogenic effects of PRMT4 in cancer cell proliferation.

In contrast to the single arginine methylation event, several sites of lysine methylation have been discovered. Munro and colleagues were the first to describe the methylation of pRB by the mono-methyl-transferase SET7/9 [28]. Methylation at K873 is important for pRB-mediated cell cycle control, as a K873A mutant had a reduced ability to repress E2F-dependent transcription. In addition, the depletion of SET7/9 in U2OS cells by siRNA treatment caused an increase in the expression of E2F target genes such as CDC6, DHFR and CDC25A, whilst no effect on these targets was observed in SAOS2 cells which lack functional pRB [28]. The K873A mutant was also compromised in its ability to induce G1 arrest in cells, suggesting a role for methylation in regulating cell cycle progression. Additionally, K873A pRB was less efficient at inducing a senescent-like phenotype when expressed in SAOS2 cells, and was compromised in its ability to induce the differentiation of mouse myoblast cell lines [28]. All three isoforms of the heterochromatin-associated protein HP1 (involved with gene silencing and transcriptional repression) appeared to bind to K873Me and HP1 was shown to act as a co-repressor in this context [29]. K873 methylation was important for the recruitment of HP1 to E2F target gene promoters under conditions of cell cycle arrest, such as in response to serum starvation and differentiation [28]. Indeed, when differentiation of mouse myoblast cells was induced, the levels of K873Me were seen to increase,



and this caused a concomitant increase in the amount of pRB and HP1 observed at E2F-responsive promoters [28].

SET7/9 can also target methylation to K810 of pRB [30]. This lysine residue constitutes the conserved basic residue in a CDK consensus site (SPXK) located around S807 and S811, and methylation of K810 by SET7/9 could block phosphorylation of these CDK sites. As pRB is predominantly hypo-phosphorylated after DNA damage, it was hypothesised that under such conditions this methylation-phosphorylation crosstalk could have an important role. Indeed, methylation of K810 was observed to increase in response to etoposide treatment which coincided with reduced phosphorylation at S807/S811 [30]. Additionally, SET7/9 depletion in cells prevented K810 methylation whilst causing a corresponding increase in global pRB phosphorylation levels. Methylation at K810 was also associated with the recruitment of pRB to E2F-responsive promoters and more efficient transcriptional repression and cell cycle arrest [30]. A further link with the DNA damage response was found when a reader for K810Me was identified as the DNA damage repair protein 53BP1 [31], which plays a role in non-homologous end-joining (NHEJ) [32]. Accordingly, 53BP1 and pRB were shown to coexist as a complex on the promoters of several E2F-responsive genes, particularly in response to DNA damage [31]. Cells lacking pRB display an increased magnitude and duration of a DNA damage response, as monitored by  $\gamma$ H2AX levels. Whilst reintroduction of pRB into RB<sup>-/-</sup> mouse embryonic fibroblasts (MEFs) suppressed this  $\gamma$ H2AX response, a K810R mutant was compromised in this ability, confirming the importance of K810 methylation during the DNA damage response [31]. Indeed, later studies have implicated pRB as having a direct role in NHEJ [33]. A recent study has suggested that JMJD3, a demethylase enzyme associated with tumour suppression and senescence [34, 35], can interact with pRB and demethylate the protein [36]. *In vitro* demethylation reactions suggested that JMJD3 could directly antagonise lysine methylation by SET7/9, and the authors proposed that JMJD3 could target the K810 site [36]. Interestingly, JMJD3 appeared to mediate a decline in both pRB methylation and phosphorylation during oncogene-induced senescence, once again highlighting possible interplay between methylation and phosphorylation events on pRB [36]. Curiously, however, JMJD3 relocalised to the cytoplasm of cells during senescence, whilst pRB is predominantly nuclear, raising questions as to the temporal and spatial regulation of pRB demethylation by JMJD3 [36].

SMYD2, another lysine mono-methyltransferase, is capable of methylating two sites in pRB, namely K810 and K860 [37, 38]. K860 methylation was detected in a number of cell types, but was found to be enhanced in several cellular processes associated with cell cycle arrest, for example in quiescent, serum starved cells, or in cells undergoing a DNA damage response [37]. K860 methylation levels also increased in a differentiating mouse myoblast cell line, though total pRB levels were also observed to rise under these conditions, suggesting that there was an accumulation of both total and methylated pRB pools [37]. A reader for the K860Me mark has been identified as the MBT domain of L3MBTL1 [37], a methyl binding protein implicated in chromatin condensation and the repression of gene expression [39]. In the context of pRB, it likely acts as a co-repressor, as it forms a complex with pRB on chromatin. This is certainly consistent with what is known about the biology of L3MBTL1 and pRB under the conditions in which the two proteins interact, and SMYD2 itself possesses growth-suppressing properties and was identified as part of a transcriptional repressor complex [40].

SMYD2 also methylates K810 (the same target for SET7/9) though the physiological conditions under which this occurs in cells are currently unknown [38]. Despite the growth inhibitory properties mentioned above, SMYD2 is observed to be overexpressed in human cancers such as bladder, breast, colon and prostate, suggesting SMYD2 can have oncogenic activity in some circumstances [38]. Indeed, depletion of SMYD2 in bladder cancer cells induced significant growth suppression. Western blot analysis of several cancer cell lines suggested a correlation between K810 methylation and pRB phosphorylation, and enhancing K810 methylation by SMYD2 overexpression resulted in a corresponding increase in phosphorylation at the neighbouring S807/S811 sites [38]. Accordingly, wild type pRB was less effective than the mutant at repressing transcription from an E2F reporter, suggesting that K810 methylation by SMYD2 encourages cell cycle progression by promoting pRB phosphorylation [38]. This is a different functional effect to that which was observed for SET7/9-mediated methylation of K810, which promoted cell cycle arrest by inhibiting pRB phosphorylation [30]. How the cell differentiates between the two K810 methylation events to mediate these opposing functions is currently unknown, though it is possible that SMYD2 methylation promotes the recruitment of a 'reader' protein other than 53BP1 that further facilitates CDK-dependent phosphorylation of pRB. What also remains unclear is the divergent roles of SMYD2 at the two sites it modifies, understanding how it is recruited to these two sites under different cellular conditions is key to unravelling the biology of these responses.

## NFκB

NFκB is a family of five (p65/RelA, RelB, c-Rel, NFκB1 and NFκB2) transcription factors involved in inflammation. Whilst the first three are activators of transcription the latter two can form inactive dimers with the others to inhibit inflammatory signalling. After toll-like-receptor, B-cell receptor or TNF receptor activation the inhibitory protein IκB is phosphorylated which results in loss of binding to NFκB. This allows NFκB dimers to migrate to the nucleus where they can activate transcription of many target genes involved in the inflammatory response such as cytokines (TNFα, IL1, IL6) chemokines (IL8, MCP-1) and regulators of apoptosis (BCL-family members, caspases) [41]. NFκB has a complex role in cancer as it can be both inhibitory and oncogenic, for a recent review see [42].

NFκB p65/RelA (for simplicity we will just refer to this subunit as NFκB from this point) is a clear example of a transcription factor being highly regulated by methylation (Figure 2C). It is symmetrically dimethylated by PRMT5 at R30, which was reported to enhance DNA binding activity [43]. PRMT5 is recruited to NFκB in response to IL-1β, a condition which stimulates NFκB activation. Indeed, two mutants, R30K and R30A, were less able to bind to target gene promoters and induce transcription after NFκB activation with IL-1β. It was proposed that this is due to a stabilising effect of dimethylation on the protein structure, which is required for subsequent DNA binding and transcriptional activation [43]. Overall this is an activating modification which results in upregulation of NFκB target genes including IL-8 and TNF-α [43]; in line with the high expression of PRMT5 in several tumour settings, this may contribute to the inflammatory response often seen in cancers.

NFκB is also methylated on several lysine residues which lead to differing outcomes: whilst methylation at K37, K218 and K221 are associated with enhanced transcriptional activity [44-46], methylation at K310, K314 and K315 are inhibitory [47, 48]. Interestingly, the first three are all present within the RHD domain, whereas the latter three are in the linker region between the RHD and the transactivation domains [46]. Mono-methylation at K37 was described by Ea and Baltimore to be SET7/9 dependent *in vitro* and *in cellulo*, as siRNA knockdown or use of a broad spectrum methyltransferase inhibitor could abrogate any signal from a western blot using a K37Me1 specific antibody [45]. Further, they showed that methylation was induced after NFκB activation by TNFα or IL-1β. Most interestingly, K37Me was revealed to alter promoter occupancy and SET7/9 depletion inhibited binding to the TNFα and IL-10 promoters, whilst there was no change observed for IκBα [45]. This is consistent with a previous study which reported that after depletion of SET7/9, only a

subset (25%) of target genes was affected [49]. This suggests that methylation at K37 can alter DNA binding specificity. Similarly, methylation at K218 and K221 by NSD1 also appeared to fine tune the NFκB response by enhancing transcription of distinct sets of target genes [44]. In this paper, methylation at K218 and K221 was shown to increase transcriptional activity and the K218R/K221A double mutant was much less active in a luciferase reporter assay. Additionally FBXL11 was identified as the demethylase for this site, and overexpression of the protein could reduce NFκB activity, as well as inhibiting cell growth and proliferation [44, 50]. In a microarray based experiment, the double mutant was unable to induce expression of 80% of genes which were activated by the wild type protein. Similar results were observed after overexpression of FBXL11. Once again, this implies that methylation in this context does not result in a general enhancement of transcriptional activity, but instead affects target gene specificity [44]. A reader for this K218/K221Me site has been found: PHF20, which contributes to enhancing NFκB activity by preventing the interaction with the inhibitory phosphatase PP2A [51]. Intriguingly, in a study which compared the effect of K37Me and K118/221Me marks on transcriptional activity, only 18 genes were observed to be shared between these distinct NFκB populations, suggesting that these modifications target a discreet set of target genes, of which only some are overlapping. In this shared pool of genes, the K218/221Q double mutant and the K37Q mutant appeared to have distinct effects and a few genes were seen to be negatively regulated by one mark, and positively by the other - for example BMP2 was down-regulated by K218/221Q, but up-regulated by K37Q [46].

The inhibitory methylation events at K314 and K315, similarly to Kontaki and Talianidis' report for lysine methylation of E2F-1, are associated with enhanced proteasome-dependent turnover [47]. SET7/9 mediated mono-methylation at both sites only occurs after TNFα stimulation of NFκB and results in inhibiting further transcription. Re-introduction of SET7/9 into cells which had been depleted of the methyltransferase resulted in attenuation of NFκB transcriptional activity. Interestingly the enhanced turnover observed after methylation appeared to target DNA bound (i.e. active) NFκB, as a mutant which could not bind DNA could also not be methylated by SET7/9 and did not undergo proteasomal degradation [47]. This implies that K314/315Me is required for switching off the NFκB response to inhibit prolonged signalling.

Mono-methylation at K310 by SETD6 is also reported to be inhibitory. Surprisingly, NFκB K310Me can be observed to be DNA bound in the nucleus of unstimulated cells, whereas it is generally

thought that NFκB remains cytoplasmic until activation. However, in this report, NFκB K310Me was shown to be present at several target gene promoters in the absence of stimulation, and could be reduced after TNFα addition. Depletion of SETD6 increased NFκB activity after stimulation, increased interleukin and cytokine secretion and increased cell growth, suggesting that the K310 methylation event was inhibitory. EHMT1 (also known as GLP1) was later identified as a reader of this methyl mark. Since EHMT1 can dimerise with EHMT2 (G9a) to facilitate H3K9 methylation (a repressive histone mark) it was suggested, and shown, that K310Me can recruit these histone methyltransferases to specific promoters to cause local chromatin remodelling and inhibit transcription. Importantly, upon stimulation with TNF, NFκB is phosphorylated at S311 which was shown to disrupt its interaction with EHMT1, resulting in enhanced transcription [48].

### **p53**

The transcription factor p53 is a key protein that orchestrates an appropriate cellular response to a variety of cellular stresses including DNA damage. Popularly referred to as the 'guardian of the genome', p53 undergoes extensive post-translational modifications following genotoxic stress, resulting in increased protein stability and translocation to the nucleus. Its numerous target genes are involved in cell cycle arrest, DNA repair and apoptosis and it is recruited to these promoters in a site-specific fashion eliciting a tumour-suppressive role (reviewed in [52, 53]).

An increasing number of studies in recent years have reported a role for p53 methylation marks during the DNA damage response (Figure 2D). Jansson *et al.* first identified arginine methylation of p53 at R333, R335 and R337 which are residues within the oligomerization domain [54]. PRMT5 was identified as the enzyme responsible for symmetric methylation at these sites, and was shown to be recruited to p53 by the stress-responsive activator of p300 (STRAP). Importantly, it was found that p53 arginine methylation formed a level of post-translational control during the DNA damage response [54]. Ectopic expression of PRMT5 led to cell cycle arrest in U2OS cells whilst depletion of PRMT5 under DNA-damage conditions sensitised cells to apoptosis in cells expressing wild-type p53 (U2OS) but not in cells lacking p53 (SAOS2). However, ectopic expression of p53 was able to sensitise PRMT5-depleted SAOS2 cells to DNA damage-induced apoptosis, implicating a role for PRMT5 in the p53 DNA damage response. p53 dependency was also observed in similar experiments performed in p53<sup>+/+</sup> and p53<sup>-/-</sup> HCT116 cells. Further, depletion of PRMT5 altered the recruitment of p53 to the promoters of some target genes: p53 occupancy was attenuated at p21, PUMA and GADD45

promoters but slightly enhanced or unaffected at p53AIP1, NOXA and APAF1 promoters [54]. This suggests that arginine methylation at R333, R335 and R337 affects target gene specificity for p53 towards cell cycle arrest rather than having a global 'on' or 'off' switch. PRMT5-dependent arginine methylation of p53 was further confirmed in a recent study and, in accordance with the previous work, was proposed to inactivate p53 pro-apoptotic function in the context of lymphomagenesis [55].

The first report of p53 lysine methylation by Chuikov *et al.* demonstrated that p53 could be methylated at K372 in the C-terminal region by SET7/9 following DNA damage [56]. K372 methylation was reported to stabilise p53 and restrict its subcellular localization to the nucleus [56]. This methylation mark was also associated with increased recruitment to, and enhanced transcription of, target genes including p21 [56]. In a manner similar to pRB, methylation has been observed to impact other modifications on p53. In this case, methylation at K372 was found to influence subsequent acetylation of surrounding residues including K373 and K382. Consequently, this caused the stabilization of p53 in response to DNA damage. This highlights the importance of crosstalk between different post-translation modifications in the p53 response [57]. A subsequent study showed that loss of SET7/9 in MEFS prevented the binding of TIP60, a chromodomain-containing protein that is known to bind methylated sites and acetylate p53 [58]. Indeed, the loss of SET7/9 prevented acetylation of murine p53 at K317, 370 and 379, demonstrating the interdependence of p53 post-translational modifications. However, there was a lack of evidence for the role of SET7/9 on the p53-dependent response to DNA damage in mice studies, suggesting the complexity of the p53 post-translational code and functional redundancy that may exist in cellular responses to stress stimuli [59, 60].

Monomethylation at K370 by SMYD2 was shown to repress p53-mediated transcription and this correlated with decreased p53 binding to the promoter of its target genes, including p21 [61]. Depletion of SMYD2 by siRNA enhanced p53-mediated apoptosis, though the precise role of K370Me1 remains to be investigated. Interestingly, the aforementioned activating p53K372Me1 modification was shown to inhibit subsequent methylation of K370, but not vice versa, indicating a one-way crosstalk in the p53 methylation code that may control p53 transcription activity [61]. There is also evidence to suggest that p53 can be dimethylated at K370, although the PKMT responsible for the dimethylation event remains unknown. Strikingly, in contrast to mono-

methylyated K370, K370Me<sub>2</sub> was found to cause the recruitment of p53 to the p21 promoter in response to DNA damage [62]. The tudor domain-containing protein 53BP1 was found to read both mono- and dimethyl K370, although there was a preference for the dimethyl modification in cells. Similarly, LSD1, an eraser of the K370Me mark, also showed a preference for the dimethyl lysine and could inhibit the interaction with 53BP1 and thereby repress transcription of p21 and MDM2 [62].

p53 can also be mono-methylated at K382 by SET8 and this was found to be a repressive mark, attenuating p53-regulated transcription of target genes [63]. Methylation at this site decreased following DNA damage and loss of this methylation mark resulted in enhanced acetylation at the same residue – the latter being a modification associated with the DNA damage response. Akin to the repressive K370Me<sub>1</sub> mark, co-expression of SET8 and p53 in p53<sup>-/-</sup> cells reduced p53 occupancy on promoters of the p53-target genes p21 and PUMA and attenuated their expression. Functionally, depletion of SET8 by siRNA sensitised cells to apoptosis and cell cycle arrest in a p53-dependent manner upon DNA damage. Notably, methylation by SET8 could increase the expression of GADD45, suggesting a change in target gene specificity as opposed to a complete ‘off’ switch [63]. A separate study alluded to a chromatin-remodelling mechanism for K382Me<sub>1</sub>-linked repression, which involved its association with L3MBTL1 in unstressed cells [64]. In a manner reminiscent to methylation at K370, p53 can also be dimethylated at K382 in response to DNA damage, although once again, no PKMT for this has yet been found. This K382Me<sub>2</sub> modification, like the K370Me<sub>2</sub> mark, has been found to be associated with stabilisation and activation of the p53 response upon DNA damage and is recognised by tudor domain-containing readers 53BP1 and PHF20 which contribute to these effects [65, 66]. The similarities between the inhibitory mono-methyl K370 and K382 and their activating dimethyl counterparts is hugely interesting and raises many questions about potential crosstalk between these marks. It would be interesting to identify the writers of the dimethyl marks and determine the timing of these in response to DNA damage – is methylation at one site required for subsequent methylation of the other?

Less is known about the methylation of p53 at K373. Homologous histone methyl-transferases EHMT1 and EHMT2 have been reported to dimethylate p53 at K373 and this was associated with the inactive form of p53 [67]. Accordingly, depletion of EHMT1 and EHMT2 by siRNA reduced methylation of K373 and sensitised cells to apoptosis [67]. At the same time, a separate study suggested a mechanism for K373 methylation-associated repression of p53 whereby MDM2

recruited histone methyl-transferase EHMT1, resulting in enhanced mono-methylation of p53 at K373 and an MDM2-dependent inhibition of p53 transcription activity [68]. Several other lysine and arginine methylation sites have also been identified throughout p53 in a mass spectrometry based study examining modifications associated with active and inactivated forms of the protein [69]. However, no further studies have yet confirmed these additional marks, or identified their writers, readers, erasers, and functional consequences.

## **Future perspectives**

Given that a large number of non-histone methylation targets have now been identified, and that many of these targets are transcription factors, cell signalling molecules, and tumour suppressors, it is unsurprising to see that deregulation of protein methylation is often linked with diseases such as cancer. Indeed, Figure 3 briefly summarises how the transcription factors described in this review function in interconnecting pathways to mediate diverse physiological outcomes such as cell cycle control, apoptosis, and inflammation. Many reports have now implicated the dysregulation of PKMTs, PRMTs and demethylase enzymes in tumorigenesis [3, 5, 7, 70]. For example, in follicular and diffuse large B-cell lymphomas, gain-of-function mutations were identified in EZH2, a H3K27-specific PKMT. These mutations cause a single amino acid substitution in the enzymatic domain of EZH2 which alters the substrate specificity of the enzyme [71]. Loss of function mutations in the PKMTs are also common and have been identified across many different cancer types, for example MLL2 and MLL3 are mutated in bladder cancer and medulloblastoma respectively [72, 73]. The PRMTs also play an important role in carcinogenesis, for example, PRMT5 is known to be over-expressed in a variety of cancers including colon, ovary, kidney, lung, bladder, liver, pancreas, breast, prostate, cervix and skin [43]. Demethylase enzymes are also frequently dysregulated in cancer, as they have both oncogenic and tumour suppressor functions in cells. For example, FBXL11 activity has been linked with the initiation and maintenance of acute myeloid leukaemia [74], whilst a number of inactivating somatic mutations in the UTX gene have been identified in multiple tumour types [75]. Whilst the effects of such mutations on the transcription program of cancer cells have been studied, focus has usually been given to linking these physiological changes to alterations in chromatin methylation levels. Given that it is becoming increasingly apparent that non-histone proteins represent major targets for methyltransferases and demethylases, it will be essential in the future to broaden our analysis beyond the chromatin, to examine the effect that dysregulated methylation of transcription factors and tumour suppressors have on tumour initiation and progression.



Given the close association between dysregulated protein methylation and diseases such as cancer, there has been great interest in the last decade to generate small molecule inhibitors to both the protein methyltransferases and the demethylases. Whilst such tools would invariably enhance our ability to study the importance of protein methylation in cell signalling events, it is their therapeutic potential that offers particular excitement. A number of inhibitors to the PKMTs, PRMTs, and demethylases have been developed, or are currently in development [70, 76, 77]. Whilst many of these compounds have yet to reach clinical trials, a number are being tested for the treatment of various cancers. For example, an EZH2 inhibitor is currently being evaluated for the treatment of advanced solid tumours and B-cell lymphomas, whilst a DOT1L inhibitor has been taken to phase 1 clinical trials for the treatment of AML and ALL [77]. The 'reader' proteins that recognise the methyl marks can also be targeted by small molecules [78], and this is currently an advancing area of research. Particular success has been achieved with members of the MBT family, where high throughput screening methods, structure-based strategies and synthetic chemistry have helped to design the first high-affinity and selective probe for L3MBTL3 [79]. Whilst inhibitors such as this undoubtedly represent a key tool for interrogating the importance of methyl-lysine and methyl-arginine readers in biological processes, their effectiveness as a therapeutic strategy is currently unknown. Still, many individual enzymes and readers that represent potential therapeutic targets lack inhibitors, and the generation of potent, selective, cell-penetrant molecules will be an area of active research for many years.

**Acknowledgements:** A.P.R. is funded by the Medical Research Council (MRC) grants 2011 (MR/J500501/1) and 2013 (MR/K501256/1). S.C. and C.C. are supported by Cancer Research U.K. Programme Award 300/A13058 and an MRC grant (to N.B.L.T.).

**Author contribution statement:** S.M.C. and A.P.R. conceived the ideas, wrote the manuscript, and created the figures. Both contributed equally to this work. C.C. also conceived ideas and wrote parts of the manuscript. N.B.L.T. discussed the subject matter and edited the manuscript.

## References:

1. Kouzarides, T. (2007) Chromatin modifications and their function, *Cell*. **128**, 693-705.
2. Tessarz, P. & Kouzarides, T. (2014) Histone core modifications regulating nucleosome structure and dynamics, *Nat Rev Mol Cell Biol*. **15**, 703-708.
3. Hamamoto, R., Saloura, V. & Nakamura, Y. (2015) Critical roles of non-histone protein lysine methylation in human tumorigenesis, *Nat Rev Cancer*. **15**, 110-124.
4. Biggar, K. K. & Li, S. S. C. (2015) Non-histone protein methylation as a regulator of cellular signalling and function, *Nat Rev Mol Cell Biol*. **16**, 5-17.
5. Yang, Y. & Bedford, M. T. (2013) Protein arginine methyltransferases and cancer, *Nat Rev Cancer*. **13**, 37-50.
6. Klose, R. J. & Zhang, Y. (2007) Regulation of histone methylation by demethylimination and demethylation, *Nat Rev Mol Cell Biol*. **8**, 307-318.
7. Johansson, C., Tumber, A., Che, K., Cain, P., Nowak, R., Gileadi, C. & Oppermann, U. (2014) The roles of Jumonji-type oxygenases in human disease, *Epigenomics*. **6**, 89-120.
8. Bottger, A., Islam, M. S., Chowdhury, R., Schofield, C. J. & Wolf, A. (2015) The oxygenase Jmjd6-a case study in conflicting assignments, *Biochem J*. **468**, 191-202.
9. Musselman, C. A., Lalonde, M. E., Cote, J. & Kutateladze, T. G. (2012) Perceiving the epigenetic landscape through histone readers, *Nat Struct Mol Biol*. **19**, 1218-27.
10. Gayatri, S. & Bedford, M. T. (2014) Readers of histone methylarginine marks, *Biochimica et biophysica acta*. **1839**, 702-10.
11. Poppy Roworth, A., Ghari, F. & La Thangue, N. B. (2015) To live or let die – complexity within the E2F1 pathway, *Molecular & Cellular Oncology*. **2**, e970480.
12. DeGregori, J., Leone, G., Miron, A., Jakoi, L. & Nevins, J. R. (1997) Distinct roles for E2F proteins in cell growth control and apoptosis, *Proc Natl Acad Sci*. **94**, 7245-50.
13. Cho, E. C., Zheng, S., Munro, S., Liu, G., Carr, S. M., Moehlenbrink, J., Lu, Y. C., Stimson, L., Khan, O., Konietzny, R., McGouran, J., Coutts, A. S., Kessler, B., Kerr, D. J. & Thangue, N. B. (2012) Arginine methylation controls growth regulation by E2F-1, *EMBO J*. **31**, 1785-97.
14. Zheng, S., Moehlenbrink, J., Lu, Y. C., Zalmas, L. P., Sagum, C. A., Carr, S., McGouran, J. F., Alexander, L., Fedorov, O., Munro, S., Kessler, B., Bedford, M. T., Yu, Q. & La Thangue, N. B. (2013) Arginine methylation-dependent reader-writer interplay governs growth control by E2F-1, *Mol Cell*. **52**, 37-51.
15. Yang, J., Valineva, T., Hong, J., Bu, T., Yao, Z., Jensen, O. N., Frilander, M. J. & Silvennoinen, O. (2007) Transcriptional co-activator protein p100 interacts with snRNP proteins and facilitates the assembly of the spliceosome, *Nucleic Acids Res*. **35**, 4485-94.
16. Valineva, T., Yang, J., Palovuori, R. & Silvennoinen, O. (2005) The transcriptional co-activator protein p100 recruits histone acetyltransferase activity to STAT6 and mediates interaction between the CREB-binding protein and STAT6, *J Biol Chem*. **280**, 14989-96.
17. Bao, X., Zhao, S., Liu, T., Liu, Y., Liu, Y. & Yang, X. (2013) Overexpression of PRMT5 Promotes Tumor Cell Growth and Is Associated with Poor Disease Prognosis in Epithelial Ovarian Cancer, *J Histochem Cytochem*. **61**, 206-217.
18. Kontaki, H. & Talianidis, I. (2010) Lysine methylation regulates E2F1-induced cell death, *Mol Cell*. **39**, 152-60.
19. Xie, Q., Bai, Y., Wu, J., Sun, Y., Wang, Y., Zhang, Y., Mei, P. & Yuan, Z. (2011) Methylation-mediated regulation of E2F1 in DNA damage-induced cell death, *Journal of receptor and signal transduction research*. **31**, 139-46.
20. Loftus, S. J., Liu, G., Carr, S. M., Munro, S. & La Thangue, N. B. (2012) NEDDylation regulates E2F-1-dependent transcription, *EMBO Rep*. **13**, 811-8.
21. Aoki, I., Higuchi, M. & Gotoh, Y. (2013) NEDDylation controls the target specificity of E2F1 and apoptosis induction, *Oncogene*. **32**, 3954-64.

22. Montenegro, M. F., Saez-Ayala, M., Pinero-Madrona, A., Cabezas-Herrera, J. & Rodriguez-Lopez, J. N. (2012) Reactivation of the tumour suppressor RASSF1A in breast cancer by simultaneous targeting of DNA and E2F1 methylation, *PLoS ONE*. **7**, e52231.
23. van den Heuvel, S. & Dyson, N. J. (2008) Conserved functions of the pRB and E2F families, *Nat Rev Mol Cell Biol*. **9**, 713-24.
24. Longworth, M. S. & Dyson, N. J. (2010) pRb, a local chromatin organizer with global possibilities, *Chromosoma*. **119**, 1-11.
25. Sherr, C. J. & McCormick, F. (2002) The RB and p53 pathways in cancer, *Cancer Cell*. **2**, 103-12.
26. Indovina, P., Marcelli, E., Casini, N., Rizzo, V. & Giordano, A. (2013) Emerging roles of RB family: new defense mechanisms against tumor progression, *J Cell Physiol*. **228**, 525-35.
27. Kim, K. Y., Wang, D. H., Campbell, M., Huerta, S. B., Shevchenko, B., Izumiya, C. & Izumiya, Y. (2015) PRMT4-mediated arginine methylation negatively regulates retinoblastoma tumor suppressor protein and promotes E2F-1 dissociation, *Mol Cell Biol*. **35**, 238-48.
28. Munro, S., Khaire, N., Inche, A., Carr, S. & La Thangue, N. B. (2010) Lysine methylation regulates the pRb tumour suppressor protein, *Oncogene*. **29**, 2357-2367.
29. Hediger, F. & Gasser, S. M. (2006) Heterochromatin protein 1: don't judge the book by its cover!, *Current opinion in genetics & development*. **16**, 143-50.
30. Carr, S. M., Munro, S., Kessler, B., Oppermann, U. & La Thangue, N. B. (2011) Interplay between lysine methylation and Cdk phosphorylation in growth control by the retinoblastoma protein, *EMBO J*. **30**, 317-27.
31. Carr, S. M., Munro, S., Zalmas, L. P., Fedorov, O., Johansson, C., Krojer, T., Sagum, C. A., Bedford, M. T., Oppermann, U. & La Thangue, N. B. (2014) Lysine methylation-dependent binding of 53BP1 to the pRb tumor suppressor, *Proc Natl Acad Sci U S A*. **111**, 11341-6.
32. Panier, S. & Boulton, S. J. (2014) Double-strand break repair: 53BP1 comes into focus, *Nat Rev Mol Cell Biol*. **15**, 7-18.
33. Cook, R., Zoumpoulidou, G., Luczynski, Maciej T., Rieger, S., Moquet, J., Spanswick, Victoria J., Hartley, John A., Rothkamm, K., Huang, Paul H. & Mitnacht, S. Direct Involvement of Retinoblastoma Family Proteins in DNA Repair by Non-homologous End-Joining, *Cell Reports*. **10**, 2006-2018.
34. Barradas, M., Anderton, E., Acosta, J. C., Li, S., Banito, A., Rodriguez-Niedenfuhr, M., Maertens, G., Banck, M., Zhou, M. M., Walsh, M. J., Peters, G. & Gil, J. (2009) Histone demethylase JMJD3 contributes to epigenetic control of INK4a/ARF by oncogenic RAS, *Genes Dev*. **23**, 1177-82.
35. Agger, K., Cloos, P. A., Rudkjaer, L., Williams, K., Andersen, G., Christensen, J. & Helin, K. (2009) The H3K27me3 demethylase JMJD3 contributes to the activation of the INK4A-ARF locus in response to oncogene- and stress-induced senescence, *Genes Dev*. **23**, 1171-6.
36. Zhao, L., Zhang, Y., Gao, Y., Geng, P., Lu, Y., Liu, X., Yao, R., Hou, P., Liu, D., Lu, J. & Huang, B. (2015) JMJD3 promotes SAHF formation in senescent WI38 cells by triggering an interplay between demethylation and phosphorylation of RB protein, *Cell Death Differ*.
37. Saddic, L. A., West, L. E., Aslanian, A., Yates, J. R., 3rd, Rubin, S. M., Gozani, O. & Sage, J. (2010) Methylation of the retinoblastoma tumor suppressor by SMYD2, *J Biol Chem*. **285**, 37733-40.
38. Cho, H. S., Hayami, S., Toyokawa, G., Maejima, K., Yamane, Y., Suzuki, T., Dohmae, N., Kogure, M., Kang, D., Neal, D. E., Ponder, B. A., Yamaue, H., Nakamura, Y. & Hamamoto, R. (2012) RB1 methylation by SMYD2 enhances cell cycle progression through an increase of RB1 phosphorylation, *Neoplasia (New York, NY)*. **14**, 476-86.
39. Trojer, P., Li, G., Sims, R. J., 3rd, Vaquero, A., Kalakonda, N., Boccuni, P., Lee, D., Erdjument-Bromage, H., Tempst, P., Nimer, S. D., Wang, Y. H. & Reinberg, D. (2007) L3MBTL1, a histone-methylation-dependent chromatin lock, *Cell*. **129**, 915-28.
40. Brown, M. A., Sims, R. J., Gottlieb, P. D. & Tucker, P. W. (2006) Identification and characterization of Smyd2: a split SET/MYND domain-containing histone H3 lysine 36-specific methyltransferase that interacts with the Sin3 histone deacetylase complex, *Molecular cancer*. **5**, 26.
41. Lawrence, T. (2009) The Nuclear Factor NF- $\kappa$ B Pathway in Inflammation, *Cold Spring Harbor Perspectives in Biology*. **1**, a001651.

42. Hoesel, B. & Schmid, J. A. (2013) The complexity of NF-kappaB signaling in inflammation and cancer, *Mol Cancer*. **12**, 86.
43. Wei, H., Wang, B., Miyagi, M., She, Y., Gopalan, B., Huang, D. B., Ghosh, G., Stark, G. R. & Lu, T. (2013) PRMT5 dimethylates R30 of the p65 subunit to activate NF-kappaB, *Proc Natl Acad Sci U S A*. **110**, 13516-21.
44. Lu, T., Jackson, M. W., Wang, B., Yang, M., Chance, M. R., Miyagi, M., Gudkov, A. V. & Stark, G. R. (2010) Regulation of NF-kappaB by NSD1/FBXL11-dependent reversible lysine methylation of p65, *Proc Natl Acad Sci U S A*. **107**, 46-51.
45. Ea, C.-K. & Baltimore, D. (2009) Regulation of NF-kB activity through lysine monomethylation of p65, *Proc Natl Acad Sci*. **106**, 18972-18977.
46. Lu, T., Yang, M., Huang, D. B., Wei, H., Ozer, G. H., Ghosh, G. & Stark, G. R. (2013) Role of lysine methylation of NF-kappaB in differential gene regulation, *Proc Natl Acad Sci U S A*. **110**, 13510-5.
47. Yang, X.-D., Huang, B., Li, M., Lamb, A., Kelleher, N. L. & Chen, L.-F. (2009) Negative regulation of NF-kB action by Set9-mediated lysine methylation of the RelA subunit, *The EMBO Journal*. **28**, 1055-1066.
48. Levy, D., Kuo, A. J., Chang, Y., Schaefer, U., Kitson, C., Cheung, P., Espejo, A., Zee, B. M., Liu, C. L., Tangsombatvisit, S., Tennen, R. I., Kuo, A. Y., Tanjing, S., Cheung, R., Chua, K. F., Utz, P. J., Shi, X., Prinjha, R. K., Lee, K., Garcia, B. A., Bedford, M. T., Tarakhovsky, A., Cheng, X. & Gozani, O. (2011) Lysine methylation of the NF-kappaB subunit RelA by SETD6 couples activity of the histone methyltransferase GLP at chromatin to tonic repression of NF-kappaB signaling, *Nature immunology*. **12**, 29-36.
49. Li, Y., Reddy, M. A., Miao, F., Shanmugam, N., Yee, J. K., Hawkins, D., Ren, B. & Natarajan, R. (2008) Role of the histone H3 lysine 4 methyltransferase, SET7/9, in the regulation of NF-kappaB-dependent inflammatory genes. Relevance to diabetes and inflammation, *J Biol Chem*. **283**, 26771-81.
50. Lu, T., Jackson, M. W., Singhi, A. D., Kandel, E. S., Yang, M., Zhang, Y., Gudkov, A. V. & Stark, G. R. (2009) Validation-based insertional mutagenesis identifies lysine demethylase FBXL11 as a negative regulator of NFkappaB, *Proc Natl Acad Sci U S A*. **106**, 16339-44.
51. Zhang, T., Park, K. A., Li, Y., Byun, H. S., Jeon, J., Lee, Y., Hong, J. H., Kim, J. M., Huang, S. M., Choi, S. W., Kim, S. H., Sohn, K. C., Ro, H., Lee, J. H., Lu, T., Stark, G. R., Shen, H. M., Liu, Z. G., Park, J. & Hur, G. M. (2013) PHF20 regulates NF-kappaB signalling by disrupting recruitment of PP2A to p65, *Nature communications*. **4**, 2062.
52. Riley, T., Sontag, E., Chen, P. & Levine, A. (2008) Transcriptional control of human p53-regulated genes, *Nat Rev Mol Cell Biol*. **9**, 402-12.
53. Murray-Zmijewski, F., Slee, E. A. & Lu, X. (2008) A complex barcode underlies the heterogeneous response of p53 to stress, *Nat Rev Mol Cell Biol*. **9**, 702-12.
54. Jansson, M., Durant, S. T., Cho, E. C., Sheahan, S., Edelmann, M., Kessler, B. & La Thangue, N. B. (2008) Arginine methylation regulates the p53 response, *Nat Cell Biol*. **10**, 1431-9.
55. Li, Y., Chitnis, N., Nakagawa, H., Kita, Y., Natsugoe, S., Yang, Y., Li, Z., Wasik, M., Klein-Szanto, A. J., Rustgi, A. K. & Diehl, J. A. (2015) PRMT5 is required for lymphomagenesis triggered by multiple oncogenic drivers, *Cancer Discov*. **5**, 288-303.
56. Chuikov, S., Kurash, J. K., Wilson, J. R., Xiao, B., Justin, N., Ivanov, G. S., McKinney, K., Tempst, P., Prives, C., Gambin, S. J., Barlev, N. A. & Reinberg, D. (2004) Regulation of p53 activity through lysine methylation, *Nature*. **432**, 353-60.
57. Ivanov, G. S., Ivanova, T., Kurash, J., Ivanov, A., Chuikov, S., Gizatullin, F., Herrera-Medina, E. M., Rauscher, F., 3rd, Reinberg, D. & Barlev, N. A. (2007) Methylation-acetylation interplay activates p53 in response to DNA damage, *Mol Cell Biol*. **27**, 6756-69.
58. Kurash, J. K., Lei, H., Shen, Q., Marston, W. L., Granda, B. W., Fan, H., Wall, D., Li, E. & Gaudet, F. (2008) Methylation of p53 by Set7/9 mediates p53 acetylation and activity in vivo, *Mol Cell*. **29**, 392-400.

59. Campaner, S., Spreafico, F., Burgold, T., Doni, M., Rosato, U., Amati, B. & Testa, G. (2011) The methyltransferase Set7/9 (Setd7) is dispensable for the p53-mediated DNA damage response in vivo, *Mol Cell*. **43**, 681-8.
60. Lehnertz, B., Rogalski, J. C., Schulze, F. M., Yi, L., Lin, S., Kast, J. & Rossi, F. M. (2011) p53-dependent transcription and tumor suppression are not affected in Set7/9-deficient mice, *Mol Cell*. **43**, 673-80.
61. Huang, J., Perez-Burgos, L., Placek, B. J., Sengupta, R., Richter, M., Dorsey, J. A., Kubicek, S., Opravil, S., Jenuwein, T. & Berger, S. L. (2006) Repression of p53 activity by Smyd2-mediated methylation, *Nature*. **444**, 629-32.
62. Huang, J., Sengupta, R., Espejo, A. B., Lee, M. G., Dorsey, J. A., Richter, M., Opravil, S., Shiekhata, R., Bedford, M. T., Jenuwein, T. & Berger, S. L. (2007) p53 is regulated by the lysine demethylase LSD1, *Nature*. **449**, 105-8.
63. Shi, X., Kachirskaya, I., Yamaguchi, H., West, L. E., Wen, H., Wang, E. W., Dutta, S., Appella, E. & Gozani, O. (2007) Modulation of p53 function by SET8-mediated methylation at lysine 382, *Mol Cell*. **27**, 636-46.
64. West, L. E., Roy, S., Lachmi-Weiner, K., Hayashi, R., Shi, X., Appella, E., Kutateladze, T. G. & Gozani, O. (2010) The MBT repeats of L3MBTL1 link SET8-mediated p53 methylation at lysine 382 to target gene repression, *J Biol Chem*. **285**, 37725-32.
65. Kachirskaya, I., Shi, X., Yamaguchi, H., Tanoue, K., Wen, H., Wang, E. W., Appella, E. & Gozani, O. (2008) Role for 53BP1 Tudor domain recognition of p53 dimethylated at lysine 382 in DNA damage signaling, *J Biol Chem*. **283**, 34660-6.
66. Cui, G., Park, S., Badeaux, A. I., Kim, D., Lee, J., Thompson, J. R., Yan, F., Kaneko, S., Yuan, Z., Botuyan, M. V., Bedford, M. T., Cheng, J. Q. & Mer, G. (2012) PHF20 is an effector protein of p53 double lysine methylation that stabilizes and activates p53, *Nat Struct Mol Biol*. **19**, 916-24.
67. Huang, J., Dorsey, J., Chuikov, S., Perez-Burgos, L., Zhang, X., Jenuwein, T., Reinberg, D. & Berger, S. L. (2010) G9a and Glp methylate lysine 373 in the tumor suppressor p53, *J Biol Chem*. **285**, 9636-41.
68. Chen, L., Li, Z., Zwolinska, A. K., Smith, M. A., Cross, B., Koomen, J., Yuan, Z. M., Jenuwein, T., Marine, J. C., Wright, K. L. & Chen, J. (2010) MDM2 recruitment of lysine methyltransferases regulates p53 transcriptional output, *EMBO J*. **29**, 2538-52.
69. DeHart, C. J., Chahal, J. S., Flint, S. J. & Perlman, D. H. (2014) Extensive post-translational modification of active and inactivated forms of endogenous p53, *Molecular & Cellular Proteomics*. **13**, 1-17.
70. McGrath, J. & Trojer, P. (2015) Targeting histone lysine methylation in cancer, *Pharmacology & Therapeutics*. **150**, 1-22.
71. Morin, R. D., Johnson, N. A., Severson, T. M., Mungall, A. J., An, J., Goya, R., Paul, J. E., Boyle, M., Woolcock, B. W., Kuchenbauer, F., Yap, D., Humphries, R. K., Griffith, O. L., Shah, S., Zhu, H., Kimbara, M., Shashkin, P., Charlot, J. F., Tcherpakov, M., Corbett, R., Tam, A., Varhol, R., Smailus, D., Moksa, M., Zhao, Y., Delaney, A., Qian, H., Birol, I., Schein, J., Moore, R., Holt, R., Horsman, D. E., Connors, J. M., Jones, S., Aparicio, S., Hirst, M., Gascoyne, R. D. & Marra, M. A. (2010) Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin, *Nat Genet*. **42**, 181-5.
72. Gui, Y., Guo, G., Huang, Y., Hu, X., Tang, A., Gao, S., Wu, R., Chen, C., Li, X., Zhou, L., He, M., Li, Z., Sun, X., Jia, W., Chen, J., Yang, S., Zhou, F., Zhao, X., Wan, S., Ye, R., Liang, C., Liu, Z., Huang, P., Liu, C., Jiang, H., Wang, Y., Zheng, H., Sun, L., Liu, X., Jiang, Z., Feng, D., Chen, J., Wu, S., Zou, J., Zhang, Z., Yang, R., Zhao, J., Xu, C., Yin, W., Guan, Z., Ye, J., Zhang, H., Li, J., Kristiansen, K., Nickerson, M. L., Theodorescu, D., Li, Y., Zhang, X., Li, S., Wang, J., Yang, H., Wang, J. & Cai, Z. (2011) Frequent mutations of chromatin remodeling genes in transitional cell carcinoma of the bladder, *Nat Genet*. **43**, 875-878.
73. Parsons, D. W., Li, M., Zhang, X., Jones, S., Leary, R. J., Lin, J. C., Boca, S. M., Carter, H., Samayoa, J., Bettegowda, C., Gallia, G. L., Jallo, G. I., Binder, Z. A., Nikolsky, Y., Hartigan, J., Smith, D. R.,

- Gerhard, D. S., Fults, D. W., VandenBerg, S., Berger, M. S., Marie, S. K., Shinjo, S. M., Clara, C., Phillips, P. C., Minturn, J. E., Biegel, J. A., Judkins, A. R., Resnick, A. C., Storm, P. B., Curran, T., He, Y., Rasheed, B. A., Friedman, H. S., Keir, S. T., McLendon, R., Northcott, P. A., Taylor, M. D., Burger, P. C., Riggins, G. J., Karchin, R., Parmigiani, G., Bigner, D. D., Yan, H., Papadopoulos, N., Vogelstein, B., Kinzler, K. W. & Velculescu, V. E. (2011) The genetic landscape of the childhood cancer medulloblastoma, *Science*. **331**, 435-9.
74. He, J., Nguyen, A. T. & Zhang, Y. (2011) KDM2b/JHDM1b, an H3K36me2-specific demethylase, is required for initiation and maintenance of acute myeloid leukemia, *Blood*. **117**, 3869-80.
75. van Haaften, G., Dalgliesh, G. L., Davies, H., Chen, L., Bignell, G., Greenman, C., Edkins, S., Hardy, C., O'Meara, S., Teague, J., Butler, A., Hinton, J., Latimer, C., Andrews, J., Barthorpe, S., Beare, D., Buck, G., Campbell, P. J., Cole, J., Forbes, S., Jia, M., Jones, D., Kok, C. Y., Leroy, C., Lin, M. L., McBride, D. J., Maddison, M., Maquire, S., McLay, K., Menzies, A., Mironenko, T., Mulderrig, L., Mudie, L., Pleasance, E., Shepherd, R., Smith, R., Stebbings, L., Stephens, P., Tang, G., Tarpey, P. S., Turner, R., Turrell, K., Varian, J., West, S., Widaa, S., Wray, P., Collins, V. P., Ichimura, K., Law, S., Wong, J., Yuen, S. T., Leung, S. Y., Tonon, G., DePinho, R. A., Tai, Y. T., Anderson, K. C., Kahnoski, R. J., Massie, A., Khoo, S. K., Teh, B. T., Stratton, M. R. & Futreal, P. A. (2009) Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer, *Nat Genet*. **41**, 521-3.
76. Maes, T., Carceller, E., Salas, J., Ortega, A. & Buesa, C. (2015) Advances in the development of histone lysine demethylase inhibitors, *Current Opinion in Pharmacology*. **23**, 52-60.
77. Kaniskan, H. Ü., Konze, K. D. & Jin, J. (2015) Selective Inhibitors of Protein Methyltransferases, *Journal of medicinal chemistry*. **58**, 1596-1629.
78. Musselman, C. A., Khorasanizadeh, S. & Kutateladze, T. G. (2014) Towards understanding methyllysine readout, *Biochimica et biophysica acta*. **1839**, 686-93.
79. James, L. I., Barsyte-Lovejoy, D., Zhong, N., Krichevsky, L., Korboukh, V. K., Herold, J. M., MacNevin, C. J., Norris, J. L., Sagum, C. A., Tempel, W., Marcon, E., Guo, H., Gao, C., Huang, X. P., Duan, S., Emili, A., Greenblatt, J. F., Kireev, D. B., Jin, J., Janzen, W. P., Brown, P. J., Bedford, M. T., Arrowsmith, C. H., Frye, S. V. (2013) Discovery of a chemical probe for the L3MBTL3 methyllysine reader domain, *Nat Chem Biol*. **9**, 184-91.
80. Avasarala, S., Van Scoyk, M., Karuppusamy Rathinam, M. K., Zerayesus, S., Zhao, X., Zhang, W., Pergande, M. R., Borgia, J. A., DeGregori, J., Port, J. D., Winn, R. A. & Bikkavilli, R. K. (2015) PRMT1 Is a Novel Regulator of Epithelial-Mesenchymal-Transition in Non-small Cell Lung Cancer, *J Biol Chem*. **290**, 13479-89.
81. Yamagata, K., Daitoku, H., Takahashi, Y., Namiki, K., Hisatake, K., Kako, K., Mukai, H., Kasuya, Y. & Fukamizu, A. (2008) Arginine Methylation of FOXO Transcription Factors Inhibits Their Phosphorylation by Akt, *Mol Cell*. **32**, 221-231.
82. Davies, C. C., Chakraborty, A., Diefenbacher, M. E., Skehel, M. & Behrens, A. (2013) Arginine methylation of the c-Jun coactivator RACO-1 is required for c-Jun/AP-1 activation.
83. Hosokawa, H., Kato, M., Tohyama, H., Tamaki, Y., Endo, Y., Kimura, M. Y., Tumes, D. J., Motohashi, S., Matsumoto, M., Nakayama, K. I., Tanaka, T. & Nakayama, T. (2015) Methylation of gata3 protein at arg-261 regulates transactivation of the il5 gene in T helper 2 cells, *J Biol Chem*. **290**, 13095-103.
84. Bandyopadhyay, S., Harris, D. P., Adams, G. N., Lause, G. E., McHugh, A., Tillmaand, E. G., Money, A., Willard, B., Fox, P. L. & Dicorleto, P. E. (2012) HOXA9 methylation by PRMT5 is essential for endothelial cell expression of leukocyte adhesion molecules, *Mol Cell Biol*. **32**, 1202-13.
85. Zhao, X., Jankovic, V., Gural, A., Huang, G., Pardani, A., Menendez, S., Zhang, J., Dunne, R., Xiao, A., Erdjument-Bromage, H., Allis, C. D., Tempst, P. & Nimer, S. D. (2008) Methylation of RUNX1 by PRMT1 abrogates SIN3A binding and potentiates its transcriptional activity, *Genes Dev*. **22**, 640-53.
86. Naeem, H., Cheng, D., Zhao, Q., Underhill, C., Tini, M., Bedford, M. T. & Torchia, J. (2007) The Activity and Stability of the Transcriptional Coactivator p/CIP/SRC-3 Are Regulated by CARM1-Dependent Methylation, *Mol Cell Biol*. **27**, 120-134.

87. Le Romancer, M., Treilleux, I., Leconte, N., Robin-Lespinasse, Y., Sentis, S., Bouchekioua-Bouzaghrou, K., Goddard, S., Gobert-Gosse, S. & Corbo, L. (2008) Regulation of estrogen rapid signaling through arginine methylation by PRMT1, *Mol Cell*. **31**, 212-21.
88. Maganti, A. V., Maier, B., Tersey, S. A., Sampley, M. L., Mosley, A. L., Ozcan, S., Pachaiyappan, B., Woster, P. M., Hunter, C. S., Stein, R. & Mirmira, R. G. (2015) Transcriptional activity of the islet beta cell factor Pdx1 is augmented by lysine methylation catalyzed by the methyltransferase Set7/9, *J Biol Chem*. **290**, 9812-22.
89. Dasgupta, M., Dermawan, J. K., Willard, B. & Stark, G. R. (2015) STAT3-driven transcription depends upon the dimethylation of K49 by EZH2, *Proc Natl Acad Sci U S A*. **112**, 3985-90.
90. Fang, L., Zhang, L., Wei, W., Jin, X., Wang, P., Tong, Y., Li, J., Du, J. X. & Wong, J. (2014) A methylation-phosphorylation switch determines Sox2 stability and function in ESC maintenance or differentiation, *Mol Cell*. **55**, 537-51.

**Table 1. Transcription factors which undergo arginine methylation**

<b>Transcription Factor</b>	<b>Site</b>	<b>SDMA/ ADMA</b>	<b>PRMT</b>	<b>Function</b>	<b>Ref</b>
E2F1	R111/113	SDMA	PRMT5	Growth promoting	[13, 14]
E2F1	R109	ADMA	PRMT1	Upregulates apoptotic target genes	[14]
p53	R333/335 /337	SDMA	PRMT5	Targets p53 to target genes associated with cell cycle arrest	[54]
TWIST1	R34	ADMA	PRMT1	Represses E-cadherin expression	[80]
NFκB (p65, RelA)	R30	SDMA	PRMT5	Upregulates NFκB dependent transcription	[43]
FOXO1	R248/250	ADMA	PRMT1	Inhibits AKT-dependent phosphorylation, enhances sensitivity to apoptosis	[81]
RAC01	R98/109	ADMA	PRMT1	Enables association with c-Jun and upregulated transcription	[82]
GATA3	R261			Alters target gene specificity	[83]
HOXA9	R140	SDMA	PRMT5	Upregulates E-selectin during inflammatory response	[84]
RUNX1	R206/210	ADMA	PRMT1	Activates transcription of target genes	[85]
p/CIP		ADMA	PRMT4	Represses transcription	[86]
ER	R260	ADMA	PRMT1	Facilitates interaction with PI3K and Src during hormone response	[87]
pRB	R787	ADMA	PRMT4	Increases phosphorylation of pRB, to drive E2F1 dependent transcription	[27]



**Table 2. Transcription factors which undergo lysine methylation**

Transcription Factor	Site	Mono or Di methyl	PKMT	Demethylase	Function	Ref
pRB	K873	Mono	SET7/9		Inhibits E2F1 dependent transcription	[28]
pRB	K810	Mono	SET7/9	JMJD3	Inhibits pRB phosphorylation and E2F1 dependent transcription, stimulates DNA repair	[30, 31, 36]
pRB	K810	Mono	SMYD2		Drives pRB phosphorylation and E2F1 dependent transcription	[38]
pRB	K860	Mono	SMYD2		Inhibits E2F1 dependent transcription	[37]
E2F1	K185	Mono	SET7/9	LSD1	Conflicting reports (see text)	[18, 19]
NFκB (p65, RelA)	K218/221	Mono/di	NSD1	FBXL11	Activates transcription of a subset of target genes	[44, 46, 50]
NFκB (p65, RelA)	K314/315	Mono/mono	SET7/9		Represses transcription of target genes, increases NFκB turnover	[46, 47]
NFκB (p65, RelA)	K37	Mono	SET7/9		Activates transcription of a subset of target genes	[45, 46]
NFκB (p65, RelA)	K310		SETD6		Represses transcription of target genes	[46, 48]
PDX1	K123/131	Mono	SET7/9		Activates transcription of target genes	[88]
STAT3	K49		EZH2		Activates transcription of IL-6 dependent target genes	[89]
SOX2	K119	Mono	SET7/9		Represses transcription of target genes, increases SOX2 turnover	[90]
p53	K372	Mono	SET7/9		Stabilises p53 during the DNA damage response	[56]
p53	K370	Mono	SMYD2	LSD1	Decrease binding to promoters, repress transcription	[61, 62]
p53	K370	Di		LSD1	Increase binding to promoters, activate transcription	[62]
p53	K382	Mono	SET8		Decrease binding to promoters, alters target gene specificity	[63, 64]
p53	K382	Di			Increase binding to promoters, activate transcription	[65, 66]
p53	K373	Di	G9a, GLP1		Represses transcription	[67]
p53	K386	Mono/di			Unknown	[65]

## Figure legends:

### Figure 1.

Representation of (A) arginine methylation by PRMTs to form mono-methyl arginine, asymmetric dimethylated arginine or symmetric dimethylated arginine and (B) lysine methylation by PKMTs to form mono-, di- or trimethyl lysine.

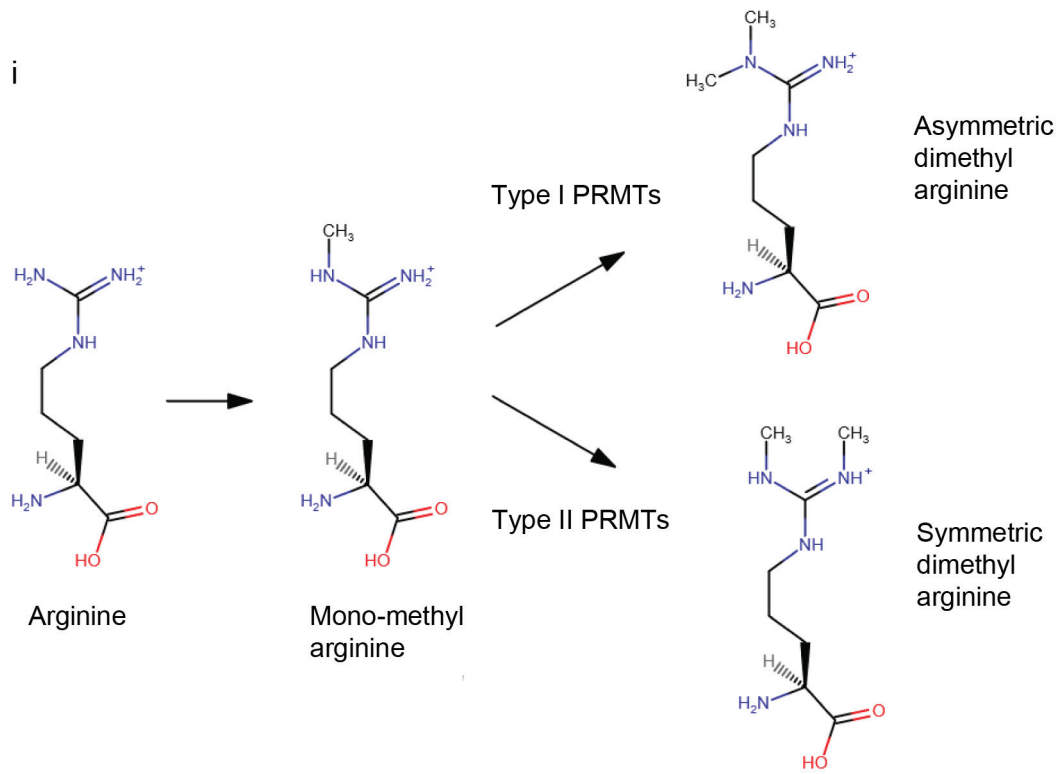
### Figure 2.

Representation of E2F-1 (A), pRB (B), NFκB (C) and p53 (D) highlighting their various protein domains and sites of arginine and lysine methylation. The 'writers', 'readers' and 'erasers' of these methyl marks are also indicated beneath each diagram, and are colour coded for clarity. For simplicity, the methyl state of each mark is not included. A, B: Retinoblastoma protein 'pocket' region; CycA: Cyclin A binding domain; DBD: DNA-binding domain; DPD: DP dimerization domain; OP: Oligomerization domain; PRD: Proline-rich domain; RD: Regulatory domain; TA1, TA2: Transcriptional activation domain 1 and 2; TAD: Transcriptional activation domain

### Figure 3.

Cell signalling pathways influenced by E2F-1, pRB, NFκB and p53. (A) Under conditions of mitogenic signalling pRB is inactivated by CDK-mediated phosphorylation events, resulting in the release of E2F-1 and the transcription of target genes associated with cell cycle progression. Under these conditions p53 is sequestered and poly-ubiquitinated by MDM2, resulting in its degradation. NFκB can also stimulate cell cycle progression via inhibition of pRB, although this is usually driven by inflammation. (B) DNA damage stimulates the activity of the sensor kinases ATM and ATR, resulting in the phosphorylation of downstream targets. This leads to the activation and stabilisation of p53 and E2F-1, which can drive cell cycle arrest or apoptosis depending on the nature and severity of the damage sustained. For example, p53 can drive expression of the CDK-inhibitor p21, which will cause pRB activation and cell cycle arrest. Alternatively, p53 and E2F-1 can both upregulate the expression of pro-apoptotic target genes or genes involved in DNA repair. During inflammation, NFκB translocates to the nucleus and can upregulate target genes involved in the inflammatory response, as well as inducing p53-dependent apoptosis under some circumstances. (C) Overview of methylation marks seen on E2F-1, pRB, NFκB and p53 in stressed and unstressed cells, and the outcome these modifications have on transcription.

i



ii

