Ablation of PRMT6 reveals a role as a negative transcriptional regulator of the p53 tumor suppressor

Mathieu Neault, Frédérick A. Mallette, Gillian Vogel, Jonathan Michaud-Levesque and Stéphane Richard*

Terry Fox Molecular Oncology Group and the Bloomfield Center for Research on Aging, Segal Cancer Centre, Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, Department of Oncology and Department of Medicine, McGill University, Montréal, Québec, Canada, H3T 1E2

Received June 25, 2012; Revised July 16, 2012; Accepted July 20, 2012

ABSTRACT
Arginine methylation of histones is a well-known regulator of gene expression. Protein arginine methyltransferase 6 (PRMT6) has been shown to function as a transcriptional repressor by methylating the histone H3 arginine 2 [H3R2(me2a)] repressive mark; however, few targets are known. To define the physiological role of PRMT6 and to identify its targets, we generated PRMT6 knockout mouse embryo fibroblasts (MEFs). We observed that early passage PRMT6−/− MEFs had growth defects and exhibited the hallmarks of cellular senescence. PRMT6−/− MEFs displayed high transcriptional levels of p53 and its targets, p21 and PML. Generation of PRMT6−/−; p53−/− MEFs prevented the premature senescence, suggesting that the induction of senescence is p53-dependent. Using chromatin immunoprecipitation assays, we observed an enrichment of PRMT6 and H3R2(me2a) within the upstream region of Trp53. The PRMT6 association and the H3R2(me2a) mark were lost in PRMT6−/− MEFs and an increase in the H3K4(me3) activator mark was observed. Our findings define a new regulator of p53 transcriptional regulation and define a role for PRMT6 and arginine methylation in cellular senescence.

INTRODUCTION
Protein arginine methylation has been shown to regulate numerous cellular processes, including transcription, DNA repair, signal transduction and RNA metabolism (1,2). There are nine protein arginine methyltransferases (PRMTs) classified into three separate families: types I, II and III depending on the type of methylation they catalyze (2,3). PRMT6 is known to be a type I enzyme that catalyzes the transfer of a methyl group from S-adenosylmethionine to the guanidino nitrogens of arginine leading to asymmetrical dimethylated arginines (4,5). PRMT6 methylates its substrates in a rapid fashion (6) and prefers arginines within a glycine–arginine-rich (GAR) motif (4). However, PRMT6 also has the ability to methylate arginines that are not within GAR rich regions, as observed in HIV Tat, Rev and nucleocapsid proteins (7–9), DNA polymerase β (10) and HMGA1 (11).

Arginine methylation has been shown to play a key role in the regulation of gene expression (12–14). It has been demonstrated that PRMT6 methylates arginine 2 of histone 3 [H3R2(me2a)] and that this mark represents a transcriptional repressive mark (15). In yeast, H3R2(me2a) interferes with the binding of SET1 methyltransferase complex to methylated H3K4 (16). In mammals, H3R2(me2a) impairs gene-activating H3K4 methylation by preventing the binding by WDR5, a subunit of the mixed lineage leukemia methyltransferase complex, consequently leading to transcriptional silencing. Thus H3R2(me2a) and H3K4(me3) are mutually exclusive histone marks (15,17,18). PRMT6 has also been observed to methylate H4R3, H2AR3 and H2AR29 (15,17–19).

The HoxA2 transcription factor, known to be sensitive to the levels of trimethylated H3K4, has been identified to be negatively regulated by PRMT6 (17). Thrombospondin-1 was identified by microarray analysis to be activated in the absence of PRMT6 causing reduced cell migration and invasion (20). PRMT6 was also shown to act as a transcriptional coactivator of progesterone, glucocorticoid and estrogen receptors coupling transcription with alternative splicing (21).
To investigate the physiological role(s) of PRMT6, we generated PRMT6-deficient mice. Although the mice appeared normal with no overt phenotype, PRMT6-deficient mouse embryonic fibroblasts (MEFs) were isolated and underwent premature senescence. This cellular state is characterized by a G0/G1 cell cycle arrest and is a mechanism that protects mammalian cells from uncontrolled growth (22). Senescence is triggered by different cellular stresses, such as DNA damage, oxidative stress, telomere shortening and activated oncogenes (23,24). In mouse cells, hallmarks of senescence include distinctive cell flattening, increased senescence-associated b-galactosidase activity (SA-b-Gal) and upregulation of the ARF-p53 pathway (25). Several targets of p53, such as the cyclin-dependent kinase (CDK) inhibitor p21, the plasminogen activator inhibitor-1 (PAI-1), GADD45a, MDM2 and promyelocytic leukemia (PML) are also transcriptionally activated upon the triggering of senescence (25–27). Cell cycle arrest is partially achieved by PAI-1, a downstream target of p53 (28,29) that acts as a negative regulator of CDK activity (30) and by the CDK inhibitor p21, an essential mediator of p53 signaling involved in cell cycle arrest (31,32). GADD45a is upregulated in a p53-dependent manner following DNA damage (33) and has been shown to partially mediate the p53 senescence response (34). PML is a p53 target that can also act upstream of p53, relocating to nuclear bodies (NBs) to amplify transcription of other p53 targets (31,35).

Herein, we describe that PRMT6 negatively regulates p53 gene expression, as PRMT6+/− MEFs display high transcriptional levels of p53 and spontaneously enter senescence. Moreover, we show that PRMT6 directly associates with the p53 promoter and locally catalyzes the methylation of the repressive mark, H3R2. These results expose the role of PRMT6 as a negative transcriptional regulator of p53 through the blockade of cellular senescence.

**MATERIALS AND METHODS**

**Generation of PRMT6−/− mice**

We constructed a PRMT6 conditional allele in mice essentially as described previously (36,37). The sequenced plasmids were linearized and electroporated into 129/Sv embryonic stem (ES) cells. Three independent ES cell clones with homologous integration at the targeting site were injected into C57BL/6J blastocysts and chimeras clones with successful germline transmission of the targeted allele were obtained. All mouse procedures were performed in accordance with McGill University guidelines, which are set by the Canadian Council on Animal Care. Genomic DNA was isolated from ear biopsies and analyzed through PCR amplification using the following primers: 5'-AGT CCA TGC TGA GCT CCG T-3' and 5'-TCC ATG CAG CTC ATA TCC A-3' to amplify an ~180 bp band in the wild-type allele and 5'-AAG GTC ACT GGA AGA AGG-3' and 5'-ACT CTC AGA ATT GCC TAG-3' to amplify the mutant allele resulting in a band of ~500 bp.

**Cell culture, cell cycle analysis and retroviral infections**

Primary MEFs were isolated from 14.5-day-postcoitum embryos following standard procedures and cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum, 1 mM sodium pyruvate and antibiotics under typical culture conditions. All flow cytometry experiments were conducted as previously described (37). Bromo-2′-deoxyuridine (BrdU) was purchased from Sigma (St Louis, MO, USA). Propidium iodide (PI) and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody were from Invitrogen (Carlsbad, CA, USA). FITC-conjugated mouse anti-BrdU monoclonal antibody was from BD Biosciences, Pharmingen (San Diego, CA, USA). Retroviral infections were achieved as described previously (38).

**Western blot**

Immunoblotting was performed as described previously (37) and antibodies used were the following: α-PRMT6 (A300-929 A, Bethyl laboratories, 1:1000); α-tubulin (B-5-1-2, Sigma, 1:50 000); α-H3R2(me2a) (NB21-1002, Novus Biologicals, 1:2000); α-H3 (ab1791, Abcam, 1:1000); α-Cyclin D1 (06-137, Millipore, 1:2000); α-p53 (2524, Cell Signaling, 1:1000); α-PML(36.1-104, Millipore, 1:1000); and α-p21 (05-345, Millipore, 1:1000).

** Immunofluorescence**

Cells growing on glass coverslips were fixed with 4% paraformaldehyde at room temperature for 15 min and permeabilized in Phosphate-buffered saline (PBS) containing 3% bovine serum albumin and 0.2% Triton X-100. Cells were then incubated with 5 μg/ml of rabbit anti-PML antibody followed by staining with an Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody and 4,6-diamidino-2-phenylindole (DAPI) staining for nuclei detection. Coverslips were mounted with Immuno-Mount purchased from Thermo Scientific. Images were taken using a Zeiss M1 fluorescence microscope.

**SA-b-gal activity**

Cells were stained for SA-b-gal activity as described (38). Briefly, MEFs were fixed with 0.5% glutaraldehyde in PBS for 15 min at room temperature. Fixed cells were washed and stained at 37°C with a staining solution containing X-gal, potassium ferrocyanide, potassium ferricyanide and MgCl2 in PBS pH 5.5.
Chromatin immunoprecipitation

MEFs were trypsinized and crosslinked with 1% paraformaldehyde for 10 min at room temperature. The fixation was quenched for 5 min with 125 mM glycine. Cells were washed twice with PBS, resuspended in hypotonic buffer (100 mM Tris, pH 7.6, 10 mM KOAc, 15 mM MgOAc, 1% IGEPAL, protease inhibitors) and fragmented by passing them 10 times through a 1 ml insulin syringe. Nuclei were pelleted at 2500g and sonicated in lysis buffer [50 mM Tris–Cl, pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% SDS, protease inhibitors]. Sonicated chromatin was diluted 5-fold in dilution buffer (16.7 mM Tris–Cl, pH 8.0, 0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 1 mM PMSF), then specific antibodies were added and incubated overnight at 4°C. Antibodies used were the following: α-IgG (Vector Laboratories, I-2000); α-PRMT6 (Bethyl laboratories, A300-928 A); α-H3R2(me2a) (Novus Biologicals, NB21-1002); α-H3K4(me3) (Millipore, 17-614); α-H3R17(me2a) (Millipore, 07-214); α-H3R26(me2a) (Millipore, 07-215); α-H3 (Abcam, ab1791). Protein A-Sepharose beads were added to immunocomplexes and incubated for 15 min with gentle rotation at room temperature. Beads were then washed twice in low-salt buffer (50 mM Tris–Cl, pH 8.0, 2 mM EDTA, 0.2% Sarkosyl), 4 times with high salt wash buffer (100 mM Tris–Cl, pH 9.0, 500 mM LiCl, 1% octylphenoxy polyethoxyethanol (IGE PAL), 1% sodium deoxycholate, 1 mM PMSF) and eluted twice with heavy Rotation buffer (100 mM Tris–Cl, pH 9.0, 500 mM LiCl, 1% SDS). NaCl was added (250 mM final concentration) to reverse cross-linking and the eluate was boiled 15 min. The samples were then incubated with 10 µg of RNase A for 30 min at 37°C and the DNA was subsequently purified using PCR purification spin columns (Qiagen).

Reverse transcription and quantitative PCR

Total RNA was isolated from wild-type or PRMT6<sup>−/−</sup> MEFs in Trizol<sup>®</sup> reagent according to the manufacturer’s protocol (Invitrogen). Five micrograms of RNA was incubated with 100 pmol of oligo dT primer at 37°C for 1 h with 100 U of M-MLV reverse transcriptase (Promega). Complementary DNA was then amplified by PCR with primers listed in Supplementary Table S1. In addition, primers for GAPDH were obtained from Qiagen (cat#: QT01658692). PCR condition were as follows: 95°C for 3 min and 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Quantitative PCR (qPCR) reactions were also performed following the QuantiTect SYBR Green PCR Kit protocol (Qiagen) using the 7500 Fast Real-Time PCR system (Applied Biosystems).

RESULTS

PRMT6<sup>−/−</sup> MEFs display features of premature senescence

We constructed a PRMT6 conditional allele (PRMT6<sup>fl/fl</sup>) in mice using a Cre-LoxP system to flank the single PRMT6 exon (Supplementary Figure S1A). Positive clones were identified using long template PCR to detect homologous recombination (Supplementary Figure S1B). Upon using Cre recombinase, PRMT6<sup>−/−</sup> mice were generated and PRMT6<sup>+/−</sup>, PRMT6<sup>−/+</sup> and PRMT6<sup>−/−</sup> MEFs isolated. To ascertain the complete removal of PRMT6, reverse transcription (RT)–PCR was preformed from total RNA isolated from PRMT6<sup>+/−</sup>, PRMT6<sup>−/+</sup> and PRMT6<sup>−/−</sup> MEFs using specific primers for the coding region of PRMT6 and GAPDH was used as a control. The expression of the PRMT6 mRNA was detected in PRMT6<sup>−/−</sup> and PRMT6<sup>−/+</sup> but not in PRMT6<sup>−/−</sup> MEFs, while GAPDH mRNA was abundant in samples from all three genotypes (Figure 1A). We next examined whether the PRMT6 protein expression was absent in PRMT6<sup>−/−</sup> MEFs and indeed extracts of PRMT6<sup>−/−</sup> and not PRMT6<sup>−/+</sup> MEFs were devoid of PRMT6, demonstrating that we generated a PRMT6 null allele (Figure 1B). As PRMT6 is known to methylate arginine 2 of histone H3 asymmetrically [H3R2(me2a)] (15–18), we investigated whether the H3R2(me2a) mark was hypomethylated in PRMT6<sup>−/−</sup> MEFs. Interestingly, we observed a reduction of H3R2(me2a) levels in PRMT6<sup>−/−</sup> MEFs compared to PRMT6<sup>−/+</sup> MEFs, but modest levels of H3R2(me2a) remained, suggesting that PRMT6 is the major enzyme that catalyzes H3R2(me2a), however additional PRMTs are also able to methylate this specific arginine residue (Figure 1B). We next examined whether the loss of PRMT6 resulted in an increased expression of other PRMTs. By immunoblotting for the major PRMTs, such as PRMT1, PRMT3, CARM1/PRMT4, PRMT5 and PRMT7, we did not observe any significant change in PRMT expression (Supplementary Figure S2).

During the culturing of primary MEFs, we noticed that PRMT6<sup>−/−</sup> MEFs displayed a significant reduction of proliferative potential at early passages (data not shown). Using flow cytometry, we observed PRMT6<sup>−/−</sup> MEFs exhibited an increase in G0/G1 and a decrease in S phase compared to PRMT6<sup>−/+</sup> MEFs (Figure 1C). These results imply a role for PRMT6 in cell proliferation and cell cycle regulation. In order to validate this assumption, we infected early passage wild-type and PRMT6<sup>−/−</sup> MEFs with retroviruses encoding the full-length myc-tagged PRMT6 or the myc-tagged catalytic inactive mutant form PRMT6<sup>VLD:KLA</sup>. The full-length PRMT6 expressing PRMT6<sup>−/−</sup> MEFs displayed a similar growth rate compared to control, whereas the cells expressing the mutant version PRMT6<sup>VLD:KLA</sup> was unable to rescue the growth phenotype (Supplementary Figure S3A). The wild-type myc-PRMT6 and myc-PRMT6<sup>VLD:KLA</sup> were expressed at similar levels in wild-type MEFs (Supplementary Figure S3B). The G0/G1 arrest was accompanied by morphological changes such as cell flattening and an enlarged cytoplasm (data not shown). This distinctive phenotype is a known hallmark of cellular senescence and is also characterized by an alteration in expression of cell cycle proteins, including an upregulation of the ARF-p53 pathway, upregulation of CDK inhibitors such as p21 and increased SA-β-Gal (25,27,39). To investigate whether ablation of PRMT6 enhanced senescence, we assessed the SA-β-Gal expression in pre-senescent PRMT6<sup>−/+</sup> or PRMT6<sup>−/−</sup> MEFs (passage 4). Indeed
**PRMT6**

_MEFs harbor H3R2me2 hypomethylation and display growth defects._ (A) Total cellular RNA was isolated from primary MEFs of the indicated genotype and subjected to semi-quantitative RT–PCR. The migration of the DNA fragments for **PRMT6** and **GAPDH** is shown. The molecular weight marker is the 1 kb ladder and the gel was visualized using ethidium bromide. No RT denotes the absence of reverse transcriptase and is a control used to show that RNA was indeed amplified and not DNA. (B) Total cellular proteins from **PRMT6**/+/+ and **PRMT6**−/− MEFs were separated by SDS–PAGE and subsequently immunoblotted with anti-PRMT6, anti-H3R2(me2a), anti-H3 and anti-α-Tubulin antibodies. (C) **PRMT6**+/+ and **PRMT6**−/− MEFs growing in log phase were trypsinized, fixed in 75% MeOH, stained with PI and analyzed by flow cytometry.

**PRMT6**−/− MEFs contained increased levels of the SA-β-Gal staining reminiscent of cellular senescence (Figure 2A).

**The p53 pathway is activated in **PRMT6**−/− MEFs**

To further evaluate the contribution of the different tumor suppressor pathways in the establishment of the premature senescence phenotype in **PRMT6**−/− MEFs, we assessed by immunoblotting the protein expression levels of p53 and two of its transcriptional targets, p21 and PML. Expression of p53, as well as p21 and PML, was increased in **PRMT6**−/− MEFs compared to wild-type MEFs (Figure 2B), suggesting that deletion of PRMT6 causes the activation of the p53 pathway. In accordance with the growth retardation phenotype, cyclin D1 was downregulated (Figure 2B). To further investigate the activation of the p53 pathway in **PRMT6**−/− MEFs, we quantified by RT–qPCR the relative levels of mRNAs of multiple p53 target genes involved in senescence as well as p53. The mRNA levels of Mdm2, PML, p21 and p53 were significantly increased in **PRMT6**−/− MEFs, while a modest increase was also observed for PAI-1 and GADD45a (Figure 2C). As expected, **PRMT6** mRNA was not detected in **PRMT6**−/− MEFs (Figure 2C). PML is a p53 target gene involved in promoting p53 transcriptional activity by recruiting it to PML NBs (31). Enhancement of PML expression as well as the number and size of PML NBs is observed in senescent cells (35). By indirect immunofluorescence, we observed a dramatic increase in PML expression accompanied by an increase in PML NBs in **PRMT6**−/− MEFs compared to **PRMT6**+/+ MEFs (Figure 2D). These data corroborate that **PRMT6**−/− MEFs are indeed prone to premature senescence and that the p53 pathway is increased in these cells.

**PRMT6 controls cellular senescence by regulating p53 expression**

p53 activity is regulated at many different levels including post-translational modifications (40,41). However, the increased p53 mRNA expression observed in **PRMT6**−/− MEFs implies that PRMT6 might directly regulate p53 at the level of transcription. We first examined whether PRMT6 associated with genomic regions of the Trp53 gene using chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed using anti-PRMT6 antibodies on wild-type MEFs and quantified for the enrichment of **PRMT6** across the different genomic regions of Trp53. Primer sets were designed such that regions of ~500 bp could be surveyed after sonication. A significant enrichment of **PRMT6** normalized to immunoglobulin G (IgG) was observed at the regions −1322 and −1062 regions of the _Trp53_ promoter. To determine specificity, we examined whether other PRMTs, such as PRMT1, PRMT4/CARM1 and PRMT5 were also enriched at these regions. By performing ChIP-qPCR experiments using PRMT1-, CARM1- and PRMT5-specific antibodies, we were unable to obtain significant enrichment along the selected _Trp53_ promoter region, suggesting that PRMT6 is specifically recruited to the _Trp53_ region (Figure 3A). **PRMT6** enrichment at the _Trp53_ −1322 region was lost in **PRMT6**−/− MEFs (Figure 3B). H3R2(me2a), mainly deposited by PRMT6 (Figure 1B), is known to inversely correlate with the H3K4(me3) activation mark at gene promoter regions and thus is linked to transcriptional repression (15,17,18). We consequently wanted to verify the status of these histone methyl marks at the _Trp53_ promoter, along with other known methylarginine marks, such as H3R17(me2a) and H3R26(me2a), to
determine whether PRMT6 could regulate p53 transcription by depositing the H3R2 repressive mark. Again, we performed a ChIP-qPCR using specific antibodies for the different methyl marks in PRMT6+/+ and PRMT6−/− MEFs. We detected a dramatic decrease in H3R2(me2a) levels and a concomitant increase of H3K4(me3) levels at the −1322 region of the Trp53 promoter (Figure 3C). As a control, we monitored the levels of H3R17(me2a) and H3R26(me2a) and they remained unchanged. These findings suggest that PRMT6 associates with the Trp53 promoter region and acts as a transcriptional repressor of p53 expression by catalyzing asymmetrical H3R2 methylation. To confirm that the premature senescence phenotype observed in p53+/+; PRMT6−/− MEFs was due to the activation of p53, we cross-bred p53+/− mice with PRMT6−/− mice to generate p53−/−; PRMT6−/− MEFs (data not shown). p53−/−; PRMT6−/− MEFs were isolated and we then performed a SA-β-Gal assay on pre-senescent MEFs (passage 4) along with wild-type, p53+/+; PRMT6−/− and p53−/−; PRMT6−/− MEFs as controls. While the expected increase in SA-β-Gal staining was observed in the p53+/+; PRMT6−/− MEFs, deletion of the Trp53 gene completely abrogated the induction of premature senescence in p53−/−; PRMT6−/− MEFs, confirming that deletion of PRMT6 triggers a p53-dependent senescence program (Figure 3D). Furthermore, the induction of the PML protein levels observed upon deletion of PRMT6 in p53+/+/PRMT6−/− was abolished in the p53−/−; PRMT6−/− MEFs (Figure 3E). Altogether, these observations identify PRMT6 as a transcriptional repressor of p53 through methylation of H3R2(me2a) at the Trp53 promoter.

PRMT6 bypasses Ras-induced senescence by regulating p53 in vivo

The p53 tumor suppressor is crucial to the establishment of the senescence program after induction by oncogenes such as activated Ras (42). RasV12, a constitutively active mutant version of Ras, is able to induce senescence in cells when expressed into low passage MEFs (43), as measured through SA-β-gal activity. To further study the role of PRMT6 in the regulation of p53, we investigated the impact of PRMT6 expression on RasV12-induced senescence. The expression of wild-type PRMT6 (PRMT6WT) allowed the bypass of RasV12-induced senescence in MEFs (Figure 4). However, a catalytically inactive mutant of PRMT6, PRMT6VLD::KLA, was unable to block RasV12-induced senescence, suggesting that PRMT6-mediated arginine methylation is a powerful negative regulator of p53.
DISCUSSION

In the present study, we have generated a conditional PRMT6 null allele in mice. Using the Cre/lox system, we show that the loss of PRMT6 expression leads to substantial reduction in arginine methylation on histone H3R2. PRMT6−/− MEFs had cell proliferation defects with cells accumulating in the G0/G1 phase of the cell cycle accompanied with dysregulation of cell cycle effectors. We furthermore demonstrated that these cells were prone to premature senescence, as visualized by flattened
post-translational modifications that modulate p53 activity are well known and characterized, but only a few proteins have been shown to regulate p53 at a transcriptional level. The Krüppel-like factor (KLF4) is a transcription factor that can regulate both positively and negatively gene expression and has been found to repress transcriptional activity of PRMT6. The KLF4, another transcriptional repressor of p53, also contributes to the bypass of RasV12-mediated senescence suggesting that the transcriptional control of p53 plays a role during tumorigenesis (55). Altogether, our results uncover PRMT6 as a novel transcriptional repressor of p53 controlling the senescence program through histone methylation.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figures 1–3.

ACKNOWLEDGEMENTS
We thank members of the Richard laboratory for discussion.

FUNDING
M.N. holds a studentship award from the Fonds de Recherche en Santé du Québec (FRSQ). P.A.M. holds postdoctoral fellowships from the FRSQ, the Canadian Institutes of Health Research (CIHR) and the Terry Fox Foundation through an award from the National Cancer Institute of Canada (NCIC). This work was funded by CIHR [MOP-93811 to S.R.], who is a recipient of a Chercheur-National Award from the FRSQ. Funding for open access charge: Canadian Institutes for Health Research [MOP-93811].

Conflict of interest statement. None declared.

REFERENCES
1. Bedford, M.T. and Richard, S. (2005) Arginine methylation an emerging regulator of protein function. Mol. Cell, 18, 263–272.
2. Bedford, M.T. and Clarke, S.G. (2009) Protein arginine methylation in mammals: who, what, and why. Mol. Cell, 33, 1–13.
3. Zarita-Lopez, C.I., Sandberg, T., Kelly, R. and Clarke, S.G. (2012) Human protein arginine methyltransferase 7 (PRMT7) is a type III enzyme forming ω-NG-monomethylated arginine residues. J. Biol. Chem., 287, 7859–7870.
4. Frankel, A., Yadav, N., Lee, J., Branscombe, T.L., Clarke, S. and Bedford, M.T. (2002) The novel human protein arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. J. Biol. Chem., 277, 3537–3543.
5. Lakowski,T.M. and Frankel,A. (2009) A kinetic study of human arginine N-methyltransferase 6 reveals a distributive mechanism. J. Biol. Chem., 283, 10015–10025.

6. Obianyi,O. and Thompson,P.R. (2012) Kinetic mechanism of protein arginine methyltransferase 6 (PRMT6). J. Biol. Chem., 17, 6062–6071.

7. Boulanger,M.C., Liang,C., Russell,R.S., Lin,R., Bedford,M.T., Wainberg,M.A. and Bedford,S. (2008) Methylation of Tat by PRMT6 regulates human immunodeficiency virus type 1 gene expression. J. Virol., 79, 124–131.

8. Invernizzi,C.F., Xie,B., Frankel,F.A., Feldhammer,M., Roy,B.B., Richard,S. and Wainberg,M.A. (2007) Arginine methylation of the HIV-1 nucleocapsid protein results in its diminished function. AIDS, 21, 795–807.

9. Invernizzi,C.F., Xie,B., Richard,S. and Wainberg,M.A. (2006) PRMT6 diminishes HIV-1 Rev binding to and export of viral RNA. Retrovirology, 3, 93.

10. El-Andaloussi,N., Valovka,T., Touelle,M., Steinacher,R., Focke,F., Gehrig,P., Covic,M., Hassa,P.O., Schar,P., Hubscher,U. et al. (2006) Arginine methylation regulates DNA polymerase beta. Mol. Cell, 22, 51–62.

11. Miranda,T.B., Webb,K.J., Edberg,D.D., Reeves,R. and Clarke,S. (2011) Histone arginine methylation in genome maintenance and cell proliferation. Mol. Cell, 43, 928–932.

12. Di Lorenzo,A. and Bedford,M.T. (2011) Histone arginine methyltransferases. Genes Dev., 25, 2830–2839.

13. Kouzarides,T. (2007) Chromatin modifications and their function. Cell, 128, 693–705.

14. Kouzarides,T. (2007) Arginine methylation at histone H3R2 methyltransferase 6 specifically methylates the nonhistone chromatin protein HMGA1a. Biochem. Biophys. Res. Commun., 363, 831–835.

15. Di Lorenzo,A. and Bedford,M.T. (2011) Histone arginine methylation. FEBS Lett., 585, 2024–2031.

16. Lee,D.Y., Trigges,C., Strahl,B.D. and Stulc,B., M.R. (2004) Role of Protein Methylation in Regulation of Transcription. Endocr. Rev., 26, 147–170.

17. Kouzarides,T. (2007) Chromatin modifications and their function. Cell, 128, 693–705.

18. Guccione,E., Bassi,C., Casadio,F., Martinato,F., Cesaroni,M., Schuchhaitz,L., Luscher,B. and Amati,B. (2007) Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive. Nature, 449, 933–937.

19. Kozmiants,A., Santos-Rosa,H., Reeves,R. and Clarke,S. (2011) Histone arginine methylation in genome maintenance and cell proliferation. Mol. Cell, 43, 928–932.

20. Hylius,D., Stein,C., Schnabel,K., Schiltz,E., Imhof,A., Dou,Y., Hsieh,J. and Bauer,U.M. (2007) PRMT6-mediated methylation of R2 in histone H3 targets H3 K4 trimethylation. Genes Dev., 21, 3369–3380.

21. Iberg,A.N., Espejo,A., Cheng,D., Kim,D., Michaud-Levesque,J., Richards,S. and Bedford,M.T. (2008) Arginine methylation of the histone tail impedes effector binding. J. Biol. Chem., 283, 3006–3100.

22. Waldmann,T., Izzo,A., Kamieniarz,K., Richter,F., Vorgler,C., Sarg,B., Lindner,H., Young,N.L., Mittler,G. and Garcia,B.A. et al. (2011) Methylation of H2A29 is a novel repression PRMT6 target. Epigenetics Chromatin, 4, 11.

23. Michaud-Levesque,J. and Richards,S. (2009) Thrombospondin-1 is a transcriptional repression target of PRMT6. J. Biol. Chem., 284, 21338–21346.

24. Harrison,M.J., Tang,Y.H. and Dowhan,D.H. (2010) Protein arginine methyltransferase 6 regulates multiple aspects of gene expression. Nucleic Acids Res., 38, 2201–2216.

25. Michaloglou,C., Vredevoije,I.C., Soengas,M.S., Denoyelle,C., Focke,F., Gehrig,P., Covic,M., Hassa,P.O., Schar,P., Hubscher,U. et al. (2006) Arginine methylation regulates DNA polymerase beta. Mol. Cell, 22, 51–62.

26. Serrano,M., Lin,A.W., McCurrach,M.E., Beach,D., Smith,J.R. and Lowe,S.W. (1994) Characterization of human Gadd45a, a p53-regulated protein. J. Biol. Chem., 269, 32672–32677.

27. Traino,D., Hoffmann,B. and Liebmann,D.A. (2006) Gadd45a suppresses Ras-driven mammalian tumorigenesis by activation of c-Jun NH2-terminal kinase and p38 stress signaling resulting in apoptosis and senescence. Cancer Res., 66, 8448–8454.

28. Ferrerey,G., de Stanchina,E., Querido,E., Bepple,M., Robins,C. and Lowe,S.W. (2000) PML is induced by oncogenic ras and promotes premature senescence. Genes Dev., 14, 2015–2027.

29. Jones,B., Bh,R., Bhat,A., Hevi,S., Baltus,G.A., Kadam,S., Zhai,H., Valdez,R. et al. (2008) Histone H3K79 methyletransferase Dot1L is essential for mammalian development and heterochromatin structure. PLoS Genet., 4, e1000190.

30. Yang,Z., Chen,T., Hebert,J., Li,E. and Richards,S. (2009) A mouse PRMT1 null allele defines an essential role for arginine methylation in genome maintenance and cell proliferation. Mol. Cell. Biol., 29, 2982–2996.

31. Mallette,F.A., Gaumont-Leclerc,M.F. and Ferrerey,G. (2007) The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence. Genes Dev., 21, 43–48.

32. Noda,A., Ning,Y., Venable,S.F., Pereira-Smith,O.M. and Smith,J.R. (1994) Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. Exp Cell Res., 211, 90–98.

33. Lujambio,A. and Lowe,S.W. (2012) The microcosmos of cancer. Nature, 482, 347–355.

34. Vigneron,A. and Vousden,K.H. (2010) p53, ROS and senescence in the control of aging. Aging, 2, 471–474.

35. Lin,A.W., Barradas,M., Stone,J.C., van Aelst,L., Serrano,M. and Lowe,S.W. (1998) Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. Genes Dev., 12, 3008–3019.

36. Serrano,M., Lin,A.W., McCurrach,M.E., Beach,D. and Lowe,S.W. (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell, 88, 593–602.

37. Rowland,B.D., Bernardes,R. and Peep,S.D. (2005) The KLF4 tumour suppressor is a transcriptional represor of p53 that acts as a context-dependent oncogene. Nat. Cell Biol., 7, 1074–1082.

38. Okoshi,R., Ando,K., Suenaga,Y., Sang,M., Kubo,N., Kizaki,H., Nakagawara,A. and Ozaki,T. (2009) Transcriptional regulation of tumor suppressor p53 by AMP-responsive element-binding protein/AMP-activated protein kinase complex in response to glucose deprivation. Genes Cells, 14, 1429–1440.
46. Raman, V., Martensen, S.A., Reisman, D., Evron, E., Odenwald, W.F., Jaffe, E., Marks, J. and Sukumar, S. (2000) Compromised HOXA5 function can limit p53 expression in human breast tumours. *Nature*, **405**, 974–978.

47. Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A.H., Schlegelberger, B., Stein, H., Dorken, B., Jenuwein, T. and Schmitt, C.A. (2005) Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature*, **436**, 660–665.

48. Chen, Z., Trotman, L.C., Shaffer, D., Lin, H.K., Dotan, Z.A., Niki, M., Koutcher, J.A., Scher, H.I., Ludwig, T., Gerald, W. *et al.* (2005) Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature*, **436**, 725–730.

49. Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A.J., Barradas, M., Benguria, A., Zaballos, A., Flores, J.M., Barbacid, M. *et al.* (2005) Tumour biology: senescence in premalignant tumours. *Nature*, **436**, 642.

50. Lin, H.K., Chen, Z., Wang, G., Nardella, C., Lee, S.W., Chan, C.H., Yang, W.L., Wang, J., Egia, A., Nakayama, K.I. *et al.* (2010) Skp2 targeting suppresses tumorigenesis by Arf-p53-independent cellular senescence. *Nature*, **464**, 374–379.

51. Ventura, A., Kirsch, D.G., McLaughlin, M.E., Tuveson, D.A., Grimm, J., Lintault, L., Newman, J., Reczek, E.E., Weissleder, R. and Jacks, T. (2007) Restoration of p53 function leads to tumour regression in vivo. *Nature*, **445**, 661–665.

52. Xue, W., Zender, L., Miething, C., Dickins, R.A., Hernando, E., Krizhanovsky, V., Cordon-Cardo, C. and Lowe, S.W. (2007) Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature*, **445**, 656–660.

53. Lim, Y., Lee, E., Lee, J., Oh, S. and Kim, S. (2008) Down-regulation of asymmetric arginine methylation during replicative and H2O2-induced premature senescence in WI-38 human diploid fibroblasts. *J. Biochem.*, **144**, 523–529.

54. Yoshimatsu, M., Toyokawa, G., Hayami, S., Unoki, M., Tsunoda, T., Field, H.I., Kelly, J.D., Neal, D.E., Machara, Y., Ponder, B.A. *et al.* (2011) Dysregulation of PRMT1 and PRMT6, Type I arginine methyltransferases, is involved in various types of human cancers. *Int. J. Cancer*, [Journal International du Cancer], **128**, 562–573.

55. Rowland, B.D. and Peeper, D.S. (2006) KLF4, p21 and context-dependent opposing forces in cancer. *Nature reviews. Cancer*, **6**, 11–23.