The arginine methyltransferase PRMT6 regulates cell proliferation and senescence through transcriptional repression of tumor suppressor genes

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ABSTRACT

The protein arginine methyltransferase 6 (PRMT6) is a coregulator of gene expression and executes its repressing as well as activating function by asymmetric dimethylation of histone H3 at R2 (H3 R2me2a). Given that elevated expression levels of PRMT6 have been reported in various cancer types, we explore here its role in cell proliferation and senescence. We find that knockdown of PRMT6 results in proliferation defects of transformed as well as non-transformed cells, causes G1-phase arrest and induces senescence. This phenotype is accompanied by transcriptional upregulation of important cell cycle regulators, most prominently the cyclin-dependent kinase (CDK) inhibitor gene p21 (p21CIP1/WAF1, CDKN1A) and p16 (p16INK4A, CDKN2A). Chromatin immunoprecipitation analysis reveals that the p21 gene is a direct target of PRMT6 and the corresponding histone mark H3 R2me2a. Using a cell model of oncogene-induced senescence (OIS), in which p21 is an essential activator of the senescent phenotype, we show that PRMT6 expression declines upon induction of senescence and conversely p21 gene expression increases. Moreover, overexpression of PRMT6 leads to reduced levels of OIS. These findings indicate that the transcriptional repressor activity of PRMT6 facilitates cell proliferation and blocks senescence by regulation of tumor suppressor genes and that this might contribute to the oncogenic capacity of PRMT6.

INTRODUCTION

Arginine methylation is an evolutionary conserved posttranslational modification, which is catalyzed by protein arginine methyltransferases (PRMTs). In mammals, these enzymes constitute a family of nine members (PRMT1-9), which share a conserved catalytic domain and perform mono- and dimethylation of the terminal guanidino nitrogens of arginine residues (1,2). Dimethylation can either be asymmetric or symmetric. A subgroup of PRMTs methylates histones as well as non-histone chromatin proteins and thereby regulates chromatin-dependent processes. Like other chromatin-modifying enzymes, PRMTs function as transcriptional coregulators and contribute either to activation or repression of gene expression (3). The enzymes themselves do not possess the capability to directly bind DNA and are recruited via interaction with transcription factors to their genomic target sites. The transcriptional functions involve PRMTs in important cellular processes, such as the regulation of cell proliferation, differentiation and apoptosis (1).

The family member PRMT6 conducts asymmetric dimethylation and prefers monomethylated arginines as substrates (4–6). In agreement with its predominant nuclear localization, the enzyme is implicated in the regulation of nuclear processes, such as DNA repair and gene expression. PRMT6 influences nucleotide excision repair by modifying the DNA polymerase β and thereby enhances the processivity of the polymerase (7). PRMT6 also plays a role in transcriptional regulation by inhibition of viral transcription and replication through methylation of the HIV transactivator protein Tat (8). Further, PRMT6 possesses histone methyltransferase activity and modifies the four core histones with histone H3

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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asymmetrically dimethylated at arginine 2 (H3 R2me2a) being the major in vivo methylation site (6,9,10). The H3 R2me2a modification contributes to transcriptional repression of HoxA genes, Myc target genes and Thrombospondin-1 (TSP1), whereas it participates in transcriptional activation of the cyclin D1 gene specifically in response to DNA-damage stimulation. H3 R2me2a accomplishes these gene regulatory functions by antagonizing H3 K4 trimethylation (H3 K4me3) and subsequent effector binding to the H3 K4me3 mark (6,9–11). In addition, it was found that PRMT6 together with PRMT4 acts as a synergistic coactivator in nuclear hormone receptor-regulated gene expression; however, the relevance of its activity toward H3 R2me2a was not addressed in this context (12).

Recent reports revealed that PRMT6 is overexpressed in several cancer types, such as breast, cervix, bladder, prostate and lung cancer, indicating that elevated levels of the enzyme might be beneficial for tumor growth and progression (13). In agreement with this, depletion of PRMT6 in a subset of tumor cell lines suppresses viability and growth (12,13). Although deregulated PRMT6 expression likely leads to an aberrant transcriptional response, which might contribute to neoplastic transformation, the relevant downstream targets of PRMT6 have so far not been described. Only the TSP1 gene, which was recently identified as a PRMT6 repressed target and which participates in prostate and lung cancer, indicating that elevated levels of PRMT6 in this model of OIS led to reduced levels of senescent phenotype. Moreover, overexpression of PRMT6 together with PRMT4 acts as a synergistic coactivator in nuclear hormone receptor-regulated gene expression; however, the relevance of its activity toward H3 R2me2a was not addressed in this context (12).

In an attempt to define the role of PRMT6 in proliferation control, we show here that depletion of PRMT6 reduces the rate of cell division, leads to cell cycle arrest and senescence. We identified the cyclin-dependent kinase inhibitor gene p21 (p21CIP1/WAF1, CDKN1A) as an important and direct downstream target of this pro-proliferative activity of PRMT6 and the corresponding histone modification H3 R2me2a. Expression of PRMT6 and p21 was found to be inversely regulated in a cell model of oncogene-induced senescence (OIS), in which p21 was revealed as a significant activator of the senescent phenotype. Moreover, overexpression of PRMT6 in this model of OIS led to reduced levels of senescence. Our findings unravel that the transcriptional repressor activity of PRMT6 is required for cell proliferation and prevents senescence by repressing p21 gene expression. Thereby, PRMT6 might contribute to an unlimited proliferation and evasion of senescence during cellular transformation.

MATERIALS AND METHODS

Cell lines and antibodies

Telomerase (hTERT)-immortalized TIG3-T cells (which express the ecotropic receptor) (14), hTERT-immortalized TIG3 BRAF-estrogen receptor (ER) cells [which express a 4-hydroxytamoxifen (4-OHT) inducible oncogenic BRAF-ER fusion] (15,16), U2OS and Phoenix cells were maintained in DMEM supplemented with 10% fetal calf serum (Gibco/BRL) at 37°C and 5% CO₂. TIG3 BRAF-ER cells were treated every 2 days with 200 nM 4-OHT (Sigma) for the indicated time periods. The following antibodies were employed: affinity-purified anti-PRMT6 rabbit serum was produced using GST-tagged proteins corresponding to amino acids 1–375 of human PRMT6, anti-p21 from Santa Cruz (sc-469), anti-p27 from Cell Signaling (3686), anti-cyclin A2 from Santa Cruz (sc-65224), anti-cyclin A2 from Santa Cruz (sc-751), anti-H3 R2me2a (asymmetric) from Millipore (07-585), anti-H3 from Abcam (ab1791), anti-CDK2 from Santa Cruz (sc-163), anti-βTubulin from Millipore (MAB3408) and rabbit immunoglobulin G (IgG) from Sigma.

Short interfering RNAs, short hairpin RNAs, transfection and retroviral infection

Short interfering RNA (siRNA) oligonucleotide duplexes were obtained from Dharmacon, Eurogentec or Sigma for targeting the human PRMT6 and p21 transcripts, respectively, or control (non-targeting) siRNAs. The following siRNA sequences were employed (sense strand indicated): sipPRMT6_1 5'-GAGCAAGACACGGACGUUU-3'; sipPRMT6_2 5'-GCAAGACCGAGGUUAUUC-3'; sipPRMT6_3 5'-GGAGGAAGAGGACUCAU-3'; sipPRMT6_4 5'-GACGUUUCAGGAGAGAUA-3'; sipPRMT6_5 5'-CGGAACAGUGGAGCUAUAU-3'; sip21 (pool of three sequences) 5'-CGAUUGGACUCUGACUUUG-3', 5'-CGGAGGACACUGACUUUG-3' and 5'-GAUGGACACUGACUUUG-3'; siLuc 5'-GAAUGUCCGGUUAUGAUUA-3'; siNon 5'-AUGAAC-3'; siScr 5'-CAUAAAGCUGAGUACUUA-3'; siGFP 5'-GCAAGCUGACCCCUGAAGUU-3'; siRNA transfections of U2OS (20 nM final concentration) were performed with the aid of Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. TIG3 BRAF-ER cells were transfected with siRNA (50 nM final concentration) using PEI or plasmid RNAiMAX (Invitrogen) according to the manufacturer's instructions. TIG3 BRAF-ER cells were treated every 2 days with 200 nM 4-OHT (Sigma) for the indicated time periods. The following antibodies were employed: affinity-purified anti-PRMT6 rabbit serum was produced using GST-tagged proteins corresponding to amino acids 1–375 of human PRMT6, anti-p21 from Santa Cruz (sc-469), anti-p27 from Cell Signaling (3686), anti-cyclin A2 from Santa Cruz (sc-65224), anti-cyclin A2 from Santa Cruz (sc-751), anti-H3 R2me2a (asymmetric) from Millipore (07-585), anti-H3 from Abcam (ab1791), anti-CDK2 from Santa Cruz (sc-163), anti-βTubulin from Millipore (MAB3408) and rabbit immunoglobulin G (IgG) from Sigma.

Western blot analysis

Cells were washed in cold PBS and subsequently lysed in IPH buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP40) for 30 min at 4°C. Debris was removed by centrifugation and protein determination of the clear lysates was performed according to Bradford. Protein extracts (20–30 μg) were analyzed by SDS–PAGE and western blot with the indicated antibodies.
Reverse transcription-quantitative PCR and chromatin immunoprecipitation-quantitative PCR

Total RNA was isolated using the RNA-Mini-Kit (Seqlab) or the RNeasy Micro Kit (Qiagen, for the small cell numbers of the colony formation assay) according to the protocol. Amounts of 10 ng–1 μg RNA were applied to reverse transcription (RT) by incubation with oligo(dT)$_{17}$ primer and 100 U M-MLV RT (Invitrogen), as recommended by the manufacturer. ChiP experiments were performed as described in (6). Complementary DNA and eluted chromatin were subjected to quantitative PCR (qPCR) analysis in triplicates with gene-specific primers. qPCR was performed using Absolute QPCR SYBR Green Mix (Thermo Scientific) and the Mx3000P real-time detection system (Agilent). For RT-qPCR, S14 or Ubiquitin Mix (Thermo Scientific) and the Mx3000P real-time detection system (Agilent). For RT-qPCR, S14 or Ubiquitin gene transcription was used for normalization. ChiP-qPCR results were expressed as percent input and additionally calculated as fold IgG. Reproducible and representative data sets are shown.

For RT-qPCR, the following primers were used: S14 forward: 5'-GGCGAGCGAGATGAATCTCA-3' and S14 reverse 5'-CAGGTTCCAGGGGTCTGGTC-3'. Ubiquitin forward: 5'-CAGTTGGTCTCGGGCTTGAG-3' and Ubiquitin reverse: 5'-CAATTGGGAATGCAACAACTTTAT-3'. PRMT6 forward: 5'-AGACACGACGCTTCCAGGAG-3' and PRMT6 reverse: 5'-CCA CTTTGTAGCCACGAG-3'. p21 forward: 5'-GGCGAGACC AGCATGACAGATTTC-3' and p21 reverse: 5'-GACGTTTCAGGAG-3'. Cyclin D1 forward: 5'-ACCAGCATGACAGATTTC-3' and Cyclin D1 reverse: 5'-GGCAGACCGAGATGAATCCTCA-3'.

For ChIP-qPCR, the following primers were used: D1, D2 forward: 5'-GGA-3' and D1, D2 reverse: 5'-GACGTTTCAGGAG-3'. Cyclin A2 forward: 5'-GACCTCCTCCTCGCACTTC-3' and cyclin A2 reverse: 5'-GTTGGGTGTCCTACTTCAA-3'.

ChIP-qPCR results were expressed as percent input and additionally calculated as fold IgG. Reproducible and representative data sets are shown.

Fluorescence-activated cell sorting assay

For quantification of the cell cycle distribution, TIG3-T cells were harvested, washed in ice-cold PBS and fixed in 80% ethanol at −20°C. After complete permeabilization, cells were washed again and DNA was stained with 54 μM propidium iodide (PI) in the presence of 38 mM sodium citrate and 250 μg/ml RNase A for 30 min at 37°C. Samples were then analyzed in a Becton Dickinson FACS Calibur and reproducible and representative data sets are shown.

Senescence-associated β-galactosidase staining

For detection of senescence, TIG3-T cells were infected, selected for 3 and 7 days later stained for senescence-associated β-galactosidase (SA-β-Gal). OIS was induced in TIG3 BRAF-ER cells upon 4-OHT treatment (200 nM) and subsequently staining for SA-β-Gal was performed at the indicated time points. As previously described (17), the enzymatic activity of SA-β-Gal was detected by using the chromogenic substrat X-Gal, which is converted at pH 6.0 to blue intracellular precipitates. The blue staining was either photographed for qualitative analysis or quantified by counting 3 × 300 cells and calculating the percentage of SA-β-Gal-positive cells.

RESULTS

PRMT6 facilitates the clonogenic growth of U2OS cells

As PRMT6 overexpression has been observed in several tumor entities (13), we asked whether the enzyme is required for survival and proliferation of transformed cells. To address this question, we employed the human osteosarcoma cell line U2OS in a clonogenic assay or so-called colony formation assay, which measures the...
ability of single cells plated in very low density to survive and to grow into colonies due to their unlimited division potential. For these studies, we established transfection of two alternative siRNAs directed against PRMT6 (siPRMT6_1 and siPRMT6_2) and one control siRNA (siLuci) in U2OS cells and achieved efficient depletion of PRMT6 protein with both PRMT6-specific siRNAs (Figure 1A). PRMT6-depleted and control U2OS cells were plated in low density and subsequently cultured for 10 days. After 10 days, colonies were stained with crystal violet and counted. In parallel, the knockdown efficiency of PRMT6 was determined on RNA level over this time period revealing that the transcript levels of PRMT6 were reduced to 25% by the two specific siRNAs compared to the control siRNA at the day of plating (0 day). The efficiency of depletion declined during 10 days of culturing, but PRMT6 mRNA levels were still reduced to 60% at final day (10 days) (Figure 1B). When counting the colonies, we found that depletion of PRMT6 with both siRNAs causes a reduction in the colony numbers of at least 60% compared to control siRNA transfected cells (Figure 1C). This suggests that PRMT6 depletion limits the proliferation capability of the cells and might lead to cell cycle arrest.

The CDK inhibitor p21 is a direct transcriptional target of PRMT6 in U2OS cells

To answer whether important cell cycle regulators, such as CDKs, CDK inhibitors and cyclins are involved in these PRMT6-dependent proliferation effects, we determined the protein levels of the CDK2, of CDK inhibitors p21 (p21\textsuperscript{CIP1/WAF1}, CDKN1A) and p27 (p27\textsuperscript{KIP1}, CDKN1B) of the CIP/KIP family and of cyclin A2 (CCNA2) in PRMT6-depleted U2OS cells. The INK4A/B locus, which encodes the CDK inhibitors of the INK4 family, was not studied, as it is inactivated by deletion and DNA methylation in U2OS cells (18,19). We employed in these siRNA experiments additional transfection controls and three additional alternative siRNA sequences targeting PRMT6. All five independent siPRMT6 sequences produced an efficient knockdown of PRMT6 on protein as well as RNA level compared to untransfected or control transfected cells (Figure 2A and B). The PRMT6 depletion was accompanied by an increase in p21 protein levels, whereas expression of CDK2 was not affected and expressions of p27 as well as cyclin A2 were not uniformly affected by the knockdown with the different siPRMT6 compared to the controls (Figure 2A). These data indicate that PRMT6 selectively influences the expression of the p21 protein.

Given that PRMT6 has a well-established function as a transcriptional coregulator (6,9–11), we next investigated by RT–qPCR analysis whether PRMT6 might affect p21 and other cell cycle regulators at the transcriptional level. In agreement with the increased p21 protein levels, depletion of PRMT6 with the different siPRMT6 led to a 2- to 5-fold increase in p21 mRNA levels in comparison to the controls (Figure 2C). Transcript levels of p27 and the third
member of the CIP/KIP family p57 (p57KIP2, CDKN1C) (Figure 2D) and of cyclin A2 and D1 (Figure 2E) were not uniformly regulated by the different siPRMT6. They were either reduced (p27, p57, cyclin A2) or slightly enhanced (cyclin D1) or not altered in comparison to the control conditions (Figure 2D and E). Taken together, these results show that PRMT6 selectively represses p21 gene transcription, but does not affect other members of the CIP/KIP family or cyclin A2 and D1.

To examine whether p21 is a direct target gene of this PRMT6-mediated repression, we performed chromatin immunoprecipitation (ChIP) analysis in U2OS cells and found that PRMT6 associates strongly (3- to 4-fold above the IgG control) with the transcriptional start site (TSS) and the transcribed region (+0.5 kb) of the p21 gene, but not with the upstream (−1 and −6.8 kb) or downstream (+8.6 kb) regions of the gene locus (Figure 2F). To elucidate whether PRMT6 recruitment coincides with the corresponding histone methyltransferase activity, the distribution of H3 R2me2a at the p21 gene locus was also determined in ChIP analysis. We found that the H3 R2me2a mark was 3-fold enriched specifically at the TSS of the p21 gene in comparison to the other regions investigated (Figure 2G). The observation that both
PRMT6 and H3 R2me2a peak at the TSS of the p21 gene indicates that p21 is a direct target gene of the H3 R2me2a-associated repressive activity of PRMT6 and might be responsible for the pro-proliferative function of PRMT6 in transformed cells.

PRMT6 facilitates cell proliferation and blocks senescence of TIG3-T cells

Next, we asked whether PRMT6 similarly controls proliferation of non-transformed cells. For these studies, we chose telomerase-immortalized TIG3-T human diploid fibroblasts and infected these cells with retroviral vectors encoding shRNAs (identical to the corresponding siRNA sequences), targeting PRMT6 or luciferase (shLuci). The three independent shRNAs directed against PRMT6 achieved an efficient depletion within a time period of up to 9 days on RNA and protein level (Figure 3A and B). When we measured population doublings for 9 days in control (empty vector), shLuci- and shPRMT6-infected cells, we found that depletion of PRMT6 leads to a considerable slowdown of cell proliferation compared to
control and shLuci infection (Figure 3C). To clarify whether this growth-inhibitory effect is caused by defective cell cycle progression, we stained the DNA of infected TIG3-T cells with PI and analyzed their cell cycle distribution by fluorescence-activated cell sorting (FACS). Similar to the population doubling analysis, the FACS profile revealed that even infection with shLuci slightly inhibits cell cycle progression, as indicated by the increased cell number in G1-phase (by 3.5%) and reduced cell number in S-phase (by 6%) compared to control-infected cells (Figure 3D). However, the effects of PRMT6 depletion were clearly more dramatic, as all three shPRMT6 constructs resulted in a 7.5–20% increased cell number in G1-phase and in a 10–15% reduced cell number in S-phase compared to the control-infected cells (Figure 3D). These findings reveal that depletion of PRMT6 gives rise to a cell cycle arrest in G1-phase. Hence, PRMT6 facilitates proliferation also in non-transformed immortalized cells.

Knowing that a continuous arrest in G1-phase is a typical feature of senescence and that some PRMT6-depleted TIG-3 cells showed a flat, enlarged senescence-like morphology (data not shown), we examined whether PRMT6 knockdown is accompanied by induction of senescence. We measured levels of SA-β-Gal, a biomarker for senescent cells. In this way, we found that the number and strength of SA-β-Gal-positive cells was significantly enhanced in the PRMT6-depleted conditions compared to the control and shLuci infections (Figure 3E). These results show that in agreement with its pro-proliferative function, PRMT6 is also crucial for suppressing the onset of senescence.

PRMT6 represses the expression of p21 as well as p16 in TIG3-T cells

To answer the question whether PRMT6 affects similar target genes that might account for the proliferation effects of PRMT6 in non-transformed cells as it does in tumor cells, we analyzed the expression of the candidate target gene p21 in shPRMT6-depleted TIG3-T cells. Additionally, as the INK4A/B locus is not mutated and as negative regulator of senescence. However, these two target genes might be differentially regulated by PRMT6 as the histone methyltransferase activity correlates with repression of p21, whereas repression of p16 is not accompanied by H3 R2me2a at the gene locus.

PRMT6 is downregulated and p21 is activated during OIS

Finally, we investigated the potential relationship between PRMT6 and p21 in a cell model of OIS, the TIG3 BRAF-ER cells. These cells express an oncogenic (constitutively active) form of BRAF, a serine/threonin-specific kinase of the MAPK pathway, fused to the ligand-binding domain of the ER. The activity of BRAF within this fusion protein is induced upon 4-OHT addition, rapidly leading to cell cycle arrest and the occurrence of senescence (15,16), which we detected within a time period of up to 6 days of 4-OHT treatment as measured by SA-β-Gal staining (Figure 5A). In this model of OIS, we found for p21 and p16, which are important and well-known inducers of senescence, that their transcript levels increase upon the onset of senescence and reach after 8 days of 4-OHT stimulation a 4.5-fold increase for p21 and a 3.5-fold increase for p16 compared to untreated TIG3 BRAF-ER cells (Figure 5B). In contrast, p27 and p14 were either reduced or only moderately enhanced in their mRNA levels during senescence induction (Figure 5C).

Knowing from our present data that p21 and p16 are both direct target genes of PRMT6, whereas p27 and p14 are not, we next asked whether expression of PRMT6 is affected after induction of OIS. We found that PRMT6 is downregulated on RNA as well as protein levels concomitantly with the establishment of the senescent cell phenotype (Figure 5D and E). After 8 days of 4-OHT stimulation, the transcript level of PRMT6 was reduced to 40% (Figure 5D). This indicates that PRMT6 and its target genes p21 and p16 are inversely regulated during OIS and that OIS-associated downregulation of PRMT6 likely allows transcriptional induction of p21 and p16.

Given that p21 shows a strong OIS-associated transcriptional upregulation (up to 4.5-fold in comparison to the untreated condition), we wished to elucidate the relevance of p21 for OIS in this cell model. To address this issue, we reduced p21 levels in TIG3 BRAF-ER cells using siRNA transfection and subsequently induced senescence with 4-OHT. Depletion of p21, which was confirmed by
RT–qPCR in comparison to the control siGFP transfection (Figure 5F), impaired the extent of OIS measured by SA-β-Gal staining (Figure 5G). These data demonstrate that p21 is essential for this senescent phenotype.

Finally, we wished to clarify whether PRMT6 is indeed important for the induction of OIS. We transfected TIG3 BRAF-ER cells with empty vector (control) or an PRMT6 overexpression construct and subsequently induced senescence by addition of 4-OHT. Overexpression of PRMT6 was confirmed by RT–qPCR (Figure 5H). We found that OIS was detectable in empty vector-transfected cells after 2 days and was further enhanced after 4 days of 4-OHT

Figure 4. The cell cycle regulators p21 and p16 are direct transcriptional targets of PRMT6 in TIG3-T cells. (A) TIG3-T cells were infected with three alternative shRNA vectors against PRMT6 (shPRMT6_1, _2, _3). The empty vector served as control. Forty-eight hours post infection cells were selected with puromycin (1 μg/ml) for 72 h. After 6 days of shRNA-mediated depletion (Day 0 corresponds to 3 days after start of selection), cells were harvested and 20 μg total protein of each sample were analyzed by western blot with the indicated antibodies. The black line indicates that the scan was cut at this point, but all stainings shown are from the same blot and exposure time. (B and C) TIG3-T cells were treated as in (A). Total RNA was prepared and analyzed by RT–qPCR for (B) p21 and p16 and (C) p14 and p15 transcript levels normalized to S14. (D–G) TIG3-T cells were harvested after 72 h and subjected to ChIP analysis using antibodies against (D and F) PRMT6 (gray bars) and corresponding control antibody (black bars, IgG rabbit) or (E and G) H3 R2me2a and corresponding control antibody (H3). Immunoprecipitated DNA was analyzed in triplicates by qPCR with primers spanning the indicated regions of the (D and E) p21 and (F and G) p16 gene locus. In (D and F), mean values were expressed as percent input of chromatin or as fold IgG, which was equated 1. In (E and G), mean values were expressed as relative enrichment compared to histone H3.
Figure 5. Effects of OIS on PRMT6 and p21 expression in TIG3 BRAF-ER cells and the relevance of PRMT6 and p21 in this model of OIS. (A) TIG3 BRAF-ER cells were treated with 4-OHT (200 nM) and stained for SA-β-Gal activity at the indicated time points. SA-β-Gal staining was photographed using a bright field microscope (left panel). The number of SA-β-Gal-positive TIG3 BRAF-ER cells was quantified (right panel). The presented result in percentage is the average from triplicate countings (each with 300 cells) ± SD. Data show a representative result of several independent experiments. (B–E) TIG3 BRAF-ER cells were treated with 4-OHT as in (A). (B–D) Total RNA was prepared and analyzed by RT-qPCR for the transcript levels of (B) p21 and p16 and (C) p27 and p14 and (D) PRMT6 at the indicated time points normalized to Ubiquitin. (E) Cells were harvested at the indicated time points and 20 μg total protein of each sample were analyzed by western blot with the indicated antibodies. (F and G) TIG3 BRAF-ER cells were transfected with siGFP or siRNAs (pool of three sequences) directed against the (continued)
treatment. Overexpression of PRMT6 reduced the levels of OIS at Days 2 and 4 when compared to the control cells (Figure 5I). Taken together, these findings identify PRMT6 as an important negative regulator of senescence. Our results suggest that the pro-proliferative and anti-senescence function of PRMT6 might be explained by its repressive function on p21 as well as p16 gene expression.

**DISCUSSION**

In the present work, we identify PRMT6 as a positive regulator of proliferation and a negative regulator of senescence. The CDK inhibitor genes p21 of the CIP/KIP family and p16 of the INK4 family were determined as the two important downstream targets, which are transcriptionally repressed by PRMT6 and responsible for mediating these cellular functions of PRMT6. The p21 protein is known to bind several CDKs, in particular CDK1 and CDK2, and their corresponding cyclins leading to inhibition of the kinase activity and interfering with activating phosphorylations of the CDKs. In contrast, p16 specifically interacts with CDK4 and CDK6 augmenting their ability to bind cyclin D and to phosphorylate and inhibit the retinoblastoma protein (20).

Both CDK inhibitors block cell cycle progression in response to a variety of stimuli; for example, mitogens, DNA-damage and oxidative stress, and promote cellular senescence. p21 achieves growth arrest in G1- as well as G2-phase of the cell cycle, whereas p16 selectively inhibits the progression through early G1 phase. The fact that PRMT6 represses transcription of both CDK inhibitor genes agrees with our observation that PRMT6-depleted cells succumb to G1 arrest, slowdown their proliferation rate and undergo senescence.

Consistently with its target genes p21 and p16, which are tumor suppressors and frequently silenced in human cancer thereby allowing deregulation of CDK activity and enforcement of cell cycle progression (21), PRMT6 has been found to be overexpressed in certain malignancies. Our findings in U2OS cells conform to reports on the positive role of PRMT6 on tumor cell survival, for example of bladder and lung cancer cells and of estrogen-stimulated breast cancer cells (12,13). Furthermore, maintenance of asymmetric dimethylation of arginines within substrate proteins correlates with highly proliferative cells (22). Accordingly, expression levels of several PRMT family members, including PRMT6, were found to be elevated in young cells and to decline in replicative and stress-induced senescent cells (22). As we found that PRMT6 transcript levels decline during OIS, the question arises by which mechanism and transcription factors gene expression of PRMT6 itself is regulated during senescence. Until now, only a few studies have dealt with the issue of how PRMT genes in general are transcriptionally controlled. A recent report identified a c-Myb binding site in the first intron of the PRMT1 gene and revealed that c-Myc is responsible for activation of PRMT1 transcription during *Xenopus* metamorphosis (23).

Interestingly, PRMT6 does not control the expression of all members of the two CDK inhibitor families. We found that p27 and p57 as well as p15 were not specifically affected in their transcript as well as protein levels by PRMT6, rather the enzyme seems selectively to affect a subset of important cell cycle regulators. Consistently, also cyclins, of which we exemplarily analyzed cyclin D1 and A2, were not altered in their expression by PRMT6. Even for the regulation of p21 and p16, we showed that they are differentially regulated by PRMT6. In case of the p21 gene locus, the PRMT6 recruitment pattern overlapped with the presence of the H3 R2me2a mark and both peak at the TSS. This indicates that the histone-modifying activity of PRMT6 might be required for its repressive activity at the p21 gene potentially by counteracting H3 K4me3 or its recognition by effector proteins (6,9,10). In contrast, although PRMT6 bound to the TSS and neighboring regions of the p16 gene, the corresponding histone mark was not enriched at these sites. Therefore, it seems possible that repression of p16 does not need the histone methyltransferase activity of PRMT6. It is conceivable that PRMT6 similar to other PRMTs modifies transcriptional coregulators, like histone deacetyltransferases, or also transcription factors, like SMADs, ER or Runt-related transcription factor 1 (RUNX1), and in this way fulfills its repressive function (3). Given that PRMT6 methylates the viral transactivator Tat, such a scenario seems possibly (8). Alternatively, PRMT6 might function as an adaptor protein and responsible for the recruitment of other effectors, which then accomplish gene silencing. So far the general importance of the catalytic activity of PRMTs in gene regulation was demonstrated only for PRMT4 (24), which leaves the possibility of a methyltransferase-independent repression by PRMT6 open.

Given that PRMT6 is not able to directly bind target genes, the question arises how PRMT6 is recruited to the
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