PRMT5 is required for cell-cycle progression and p53 tumor suppressor function

A. Scoumanne, J. Zhang and X. Chen*

Center for Comparative Oncology, University of California at Davis, Davis, CA 95616, USA

ABSTRACT

Protein arginine methyltransferases (PRMTs) mediate the transfer of methyl groups to arginines in proteins involved in signal transduction, transcriptional regulation and RNA processing. Tumor suppressor p53 coordinates crucial cellular processes, including cell-cycle arrest and DNA repair, in response to stress signals. Post-translational modifications and interactions with co-factors are important to regulate p53 transcriptional activity. To explore whether PRMTs modulate p53 function, we generated multiple cell lines in which PRMT1, CARM1 and PRMT5 are inducibly knocked down. Here, we showed that PRMT5, but not PRMT1 or CARM1, is essential for cell proliferation and PRMT5 deficiency triggers cell-cycle arrest in G1. In addition, PRMT5 is required for p53 expression and induction of p53 targets MDM2 and p21 upon DNA damage. Importantly, we established that PRMT5 knockdown prevents p53 protein synthesis. Furthermore, we found that PRMT5 regulates the expression of translation initiation factor eIF4E and growth suppression mediated upon PRMT5 knockdown is independent of p53 but is dependent on eIF4E. Taken together, we uncovered that arginine methyltransferase PRMT5 is a major pro-survival factor regulating eIF4E expression and p53 translation.

INTRODUCTION

The tumor suppressor p53 is one of the most frequently mutated genes in human cancers and p53 germline mutations are responsible for the high incidence of tumors found in Li-Fraumeni syndrome patients (1). The p53 protein functions mainly as a transcription factor regulating important cellular processes, such as cell proliferation, cell-cycle arrest, DNA repair and apoptosis in response to stress signals (2,3). In unstressed conditions, p53 is maintained at a low level in the cell due to binding of E3 ubiquitin ligase MDM2 (mouse double minute 2),

which leads to p53 ubiquitination and rapid degradation by the 26S proteasome (4). In response to DNA damage and other types of cellular stresses, sensor kinases are activated and mediate the phosphorylation of p53, which releases MDM2 and promotes p53 stabilization (5). The transcriptional activity of p53 at distinct target gene promoters is further modulated through diverse p53 post-translational modifications and interactions with co-factors (6). P53 is one of few non-histone proteins regulated through lysine methylation. Indeed, histone lysine methyltransferases KMT5, KMT3C and KMT5A methylate lysines 372, 370 and 382 in p53 C-terminus (7–9). Lysine methylation enhances or suppresses p53 transcriptional activity depending on the methylation site (10). Furthermore, our earlier study indicated that protein methyltransferases, especially arginine methyltransferases, play a role in differential target gene regulation by p53 (11).

Arginine methylation is an important process and involved in the regulation of gene expression, RNA metabolism and protein function (12,13). Protein arginine methyltransferases (PRMTs) use *S*-adenosyl-L-methionine as methyl donor to catalyze the transfer of a methyl group to arginine residues in a variety of proteins, including histones H3 and H4, Stat1 and hnRNPs (12). All PRMTs catalyze the formation of monomethylated arginines. In addition, type I PRMTs can mediate the transfer of a second methyl group to monomethylated arginines asymmetrically, and type II PRMTs can mediate the transfer of a second methyl group symmetrically (14). Most of the 11 PRMTs identified to date are type I PRMTs, including PRMT1 and coactivator-associated arginine methyltransferase-1 (CARM1). Type II PRMTs are PRMT5, PRMT7 and PRMT9. The methyltransferase activity of PRMT2, PRMT10 and PRMT11 has yet to be determined. Although all PRMTs contain a conserved catalytic core, they varied in length and structure at N- and C-termini. Several recent studies have found that methylation of histones H3 and H4 by PRMTs plays an important role in the regulation of gene expression. Indeed, methylation on histones H4R3 and H3R17 by PRMT1 and CARM1, respectively, is involved in nuclear receptor-mediated transcriptional activation (13,15,16).

*To whom correspondence should be addressed. Tel: +1 530 754 8404; Fax: +1 530 752 6042; Email: xbchen@ucdavis.edu

In contrast, methylation on histone H3R8 by PRMT5, a component of hSWI/SNF and NURD chromatin-remodeling complexes, mediates transcriptional repression of cell-cycle regulator and tumor suppressor genes, including cyclin E, ST7 and NM23 (17,18). In addition, PRMT5 plays a role in androgen receptor-driven transcription, in a manner independent of its methyltransferase activity (19). To date, it remains unclear whether PRMTs are implicated in the cellular response to stress signals, such as DNA damage and hypoxia. However, p53 has been reported to interact with PRMT1 and CARM1 *in vivo* (20). CARM1 and PRMT1 were suggested to act as co-activators of p53 involved in methylation of histones surrounding its target gene GADD45. In addition, methylation of arginines in p53 oligomerization domain by PRMT5 was recently reported to regulate p53 function (21).

In this study, we explored the role of selected PRMTs in p53 tumor suppressive function. We generated multiple stable MCF7 cell lines, which inducibly express shRNA targeting PRMT1, CARM1 or PRMT5. We found that knockdown of PRMT5, but not PRMT1 or CARM1, induces G1 arrest and inhibits cell proliferation. Importantly, we also found that PRMT5 knockdown reduces p53 expression and prevents p53 stabilization in response to DNA damage, leading to a decreased induction of p53 target genes MDM2 and p21. Consistent with this, PRMT5 deficiency inhibits mutant p53 expression. Furthermore, we revealed that PRMT5 is required for p53 protein synthesis. We showed that PRMT5 knockdown inhibits the expression of eukaryotic translation initiation factor 4E (eIF4E), an important mediator of protein translation and that eIF4E inhibition is responsible for growth suppression upon PRMT5 knockdown. Therefore, we suggest that PRMT5, a class II arginine methyltransferase, plays an essential role for cell survival through regulating eIF4E expression and p53 translation.

MATERIALS AND METHODS

Reagents

Nutlin-3 was purchased from Cayman Chemical Company. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-p21, anti-MDM2 (SMP14), anti-p53 and anti-eIF4E antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-hemagglutinin (HA) and anti-MDM2 (AB-2) were from Covance (Berkeley, CA) and EMB Biosciences (San Diego, CA), respectively. Anti-PRMT5 and anti-CARM1 antibodies were from Upstate (San Diego, CA). Anti-PRMT1 was from Abcam (Cambridge, MA). Other reagents were from Sigma (St Louis, MO).

Plasmids

To generate PRMT5 shRNA vector, oligonucleotides (5′-GAT CCC **CAC CGC TAT TGC ACC TTG GA**T TCA AGA GA**T CCA AGG TGC AAT AGC GGT** TTT TTG GAA A-3′ and 5′-AGC TTT TCC AAA AAA **CCG CTA TTG CAC CTT GGA** TCT CTT GAA **TCC AAG GTG CAA TAG CGG T**GG G-3′) were

designed to target PRMT5 nucleotides 1689-1707 (shown in boldface). To generate CARM1 shRNA vector, oligonucleotides (5′-GAT CCC **CAC GGC GAG ATC AG CGG CA**T TCA AGA GA**T GCC GCT GGA TCT CGC CGT** TTT TTG GAA A-3′ and 5′-AGC TTT TCC AAA AAA **CGG CGA GAT CCA GCG GCA** TCT CTT GAA **TGC CGC TGG ATC TCG CCG T**GG G-3′) were designed to target CARM1 nucleotides 257-275 (shown in boldface). To generate PRMT1 shRNA vector, oligonucleotides (5′-GAT CCC **CAG CCC AAC GCT GAG GAC AT**T TCA AGA GAA **TGT CCT CAG CGT TGG GCT** TTT TTG GAA A-3′ and 5′-AGC TTT TCC AAA AAA **GCC CAA CGC TGA GGA CAT** TCT CTT GAA **ATG TCC TCA GCG TTG GGC TG**G G-3′) were designed to target PRMT1 nucleotides 110-128 (shown in boldface). Oligonucleotides were annealed and cloned into pTER, a PolIII promoter-driven shRNA expression vector. Resulting plasmids were named pTER/PRMT5, pTER/CARM1 and pTER/PRMT1, respectively. To stably express siRNA against p53, the pBabe-U6-sip53 construct was used as described previously (22). To transiently knockdown PRMT5, double-stranded RNA oligos (ACC GCU AUU GCA CCU UGG AdTdT) and scrambled siRNA were synthesized by Dharmacon (Chicago, IL).

Cell culture

The MCF7-pTR-7 cell line was generated previously by transfection of MCF7 cells with pcDNA6 vector that expresses a tetracycline repressor (11). To generate MCF7 cell lines that inducibly express PRMT5, CARM1 and PRMT1 shRNAs, MCF7-pTR-7 cells were transfected with pTER/PRMT5, pTER/CARM1 or pTER/PRMT1, and the pBabe vector for puromycin selection. To generate MCF7 cell lines that stably express p53 shRNA and inducibly express PRMT5 shRNA, MCF7-pTR-7 cells were transfected with pBabe-U6-sip53 and pTER/PRMT5. Individual clones were screened for inducible knockdown of the target gene by western blot analysis and two representative clones were chosen for subsequent studies. Human colon carcinoma RKO and HCT116 cells, colon adenocarcinoma SW480 cells and glioblastoma T98G cells were obtained from ATCC (Manassas, VA) and cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 8% fetal bovine serum (Hyclone, Logan, UT).

Western blot analysis

Cells are washed twice with PBS, re-suspended with 2× SDS sample buffer, incubated at 95°C for 5 min, and used for western blot analysis as previously described (23).

Growth rate and colony formation assay

To determine cell growth rate, 1×10^4 cells were seeded per 6-well plate. After 24 h, cells were untreated or treated with tetracycline. At times indicated, cells were collected and counted using a Coulter cell Counter (Coulter Corporation). For colony formation assay, cells seeded at 1×10^3 cells per well in a 6-well plate were incubated in the absence or presence of tetracycline for 10 days.

Cells were fixed with fixative for 10 min and then stained with crystal violet for 20 min as previously described (24).

DNA histogram analysis

For DNA histogram analysis, 2×10^5 cells were seeded per 90 mm-diameter plate and were untreated or treated with tetracycline for 4 days. Cells were collected and stained with propidium iodide as previously described, and examined by fluorescence-activated cell sorter (FACS Calibur) (25).

[^{35}S]Methionine labeling

For ^{35}S protein labeling, 0.5×10^6 cells were seeded per 90 mm-diameter plate, untreated or treated with tetracycline for 3 days. After 2 washes and incubation with Methionine-free DMEM (Invitrogen, Carlsbad, CA) for 3 h, cells were incubated with [^{35}S]methionine-containing DMEM (50 μCi/ml) for 30 min (Express ^{35}S protein assay labeling mix, Perkin Elmer). Cells extracts were prepared in 0.5% NP-40 lysis buffer as described previously (24).

p53 immunoprecipitation and autoradiography

Immunoprecipitation was performed as previously described (24). [^{35}S]methionine-labeled cells extracts were prepared in 0.5% NP-40 lysis buffer and p53 was immunoprecipitated with an anti-p53 antibody or control rabbit IgG at 4°C overnight. After 4 washes with 0.5% NP-40 lysis buffer, immunoprecipitated p53 was resuspended with 2× SDS sample buffer, incubated at 95°C for 5 min, and used for SDS PAGE. Gels were fixed with fixation solution (10% acetic acid and 30% methanol), incubated with Enhancer (Perkin Elmer, Boston, MA) for 30 min and washed three times with distilled water. Gels were dried using a gel dryer and vacuum pump (Biorad, Hercules, CA) and exposed to X-ray film at −80°C.

Statistical analysis

Unless otherwise stated, all experiments were performed in triplicates and data are represented as mean ± SD. Two group comparisons were analyzed by two-tailed Student's *t*-test. *P*-values were calculated, and values ≤0.05 were considered significant.

RESULTS

Knockdown of PRMT5 inhibits cell proliferation and induces G1 arrest

To determine whether a deficiency in PRMT5 arginine methyltransferase has an effect on cell proliferation, we generated MCF7 cell lines, in which PRMT5 is inducibly knocked down by the tetracycline-inducible shRNA expression system. Three representative MCF7-PRMT5-KD cell lines, MCF7-PRMT5-KD-41/-62/-72, along with parental MCF7-pTR-7 cell line, are shown in Figure 1A. We showed that PRMT5 levels were decreased by 55% in MCF7-PRMT5-KD-41 cells and 35% in MCF7-PRMT5-KD-62/-72 cells upon the induction of PRMT5 shRNA for 4 days. The level of GAPDH was determined as a

loading control. Next, we performed a colony-formation assay to determine the long-term effect of PRMT5 knockdown on cell proliferation (Figure 1B). In the parental MCF7-pTR-7 cell line, tetracycline was not found to have any effect on MCF7 cell proliferation (Figure 1B, the first column). In contrast, we found that the ability of MCF7 cells to proliferate and form colonies was clearly reduced upon knockdown of PRMT5 for 10 days. To further characterize the short-term impact of PRMT5 knockdown on cell proliferation, we performed an 8-day growth curve on MCF7-pTR-7 and MCF7-PRMT5-KD-41/-62/-72 (Figure 1C). We found that there was a significant decrease in the number of proliferating cells starting after 4 days of PRMT5 knockdown. Thus, to characterize the cell-cycle profile of PRMT5 knockdown cells, DNA histogram assay was performed on MCF7-pTR-7 and MCF7-PRMT5-KD-41/-62/-72 cells untreated or treated with tetracycline for 5 days (Figure 1D). We found that the number of cells in G1 was significantly increased upon knockdown of PRMT5, concomitantly with a decrease in the number of cells in S phase. Taken together, these data suggest that PRMT5 is required for cell-cycle progression.

To determine whether the expression of an exogenous PRMT5 has an effect on MCF7 cell proliferation, we generated cell lines in which N-terminally HA-tagged PRMT5 is inducibly expressed under the control of a tetracycline-regulated promoter (data not shown). Next, we performed colony-formation assays and found that the ability of MCF7 to proliferate and form colonies was not affected by PRMT5 expression for 10 days. These results suggest that overexpression of PRMT5 arginine methyltransferase alone has no effect on cell proliferation.

Knockdown of CARM1 and PRMT1 has no effect on cell proliferation

To determine whether a deficiency in CARM1 or PRMT1 has an effect on cell proliferation, we generated MCF7 cell lines that inducibly express shRNA targeting CARM1 or PRMT1 in the tetracycline-inducible shRNA expression system. Representative cell lines for the inducible knockdown of CARM1 (MCF7-CARM1-KD-12/-17) and PRMT1 (MCF7-PRMT1-KD-6), along with the parental MCF7-pTR-7 cell line, are shown in Figure 2A. We found that levels of CARM1 and PRMT1 were decreased by at least 50% upon induction of their respective shRNA for 4 days. Next, we performed a colony-formation assay to determine the long-term effect of CARM1 and PRMT1 knockdown on cell proliferation (Figure 2B). We found that the ability of MCF7-CARM1-KD-12/-17 cells to proliferate was only slightly attenuated upon knockdown of CARM1 for 10 days. Similarly, the proliferation of MCF7-PRMT1-KD-6 cells was slightly decreased upon knockdown of PRMT1. We further characterized this effect and found that the total number of colonies was not affected by knockdown of CARM1 or PRMT1. In addition, we found that the number of small colonies (less than 1 mm in diameter) was not significantly affected by knockdown of CARM1 or PRMT1 (Figure 2C).

Since CARM1 and PRMT1 belong to type I PRMTs, they may have redundant effects on cell proliferation.
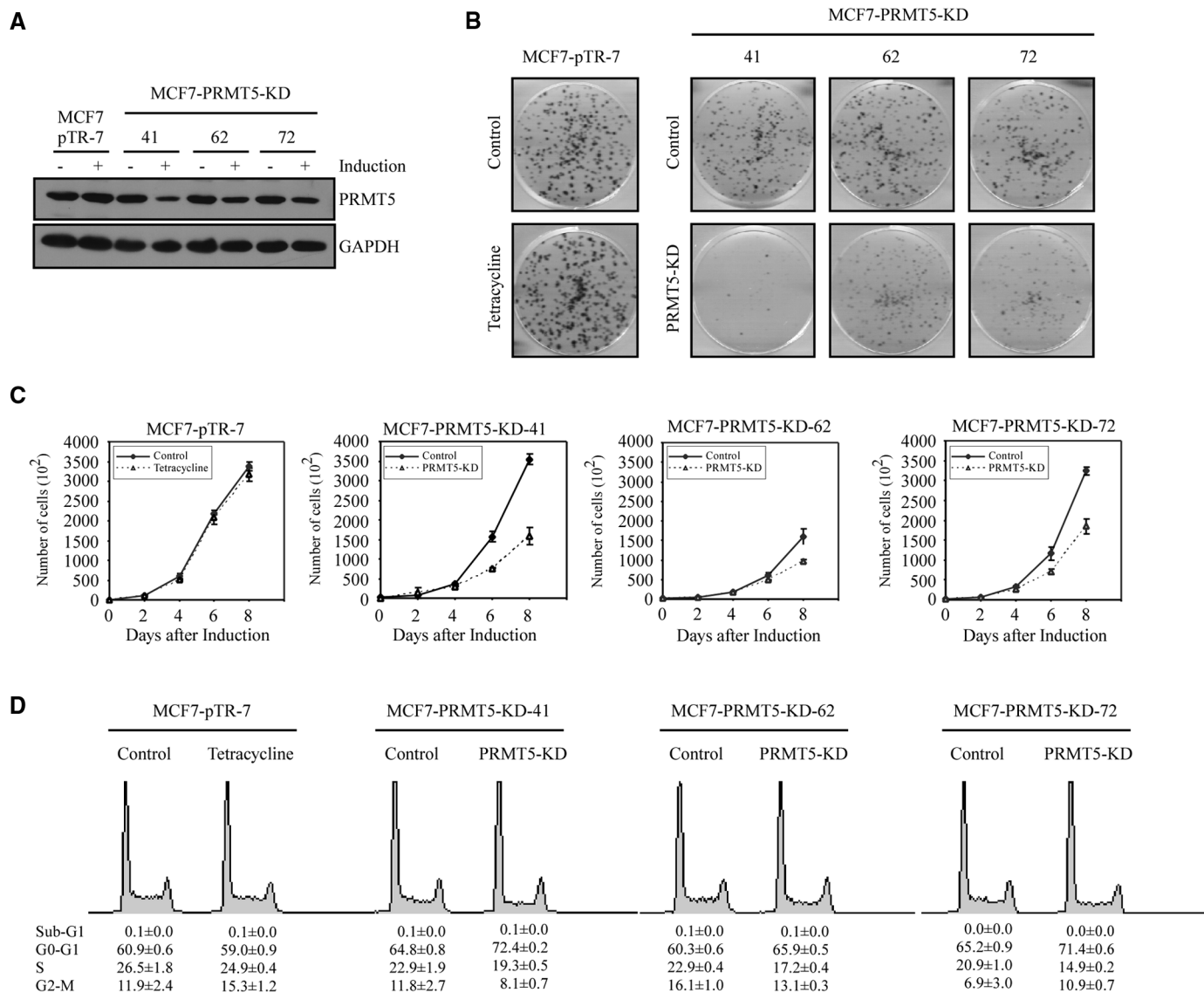
Figure 1. PRMT5 is required for G1-S transition. (**A**) Generation of MCF7 cell lines inducibly expressing PRMT5 shRNA. Levels of PRMT5 and GAPDH were assayed in MCF7-pTR-7 and MCF7-PRMT5-KD-41/-62/-72 cells cultured in the absence (−) or presence (+) of tetracycline for 4 days. (**B**) PRMT5 is required for colony formation. MCF7-pTR-7 and MCF7-PRMT5-KD-41/-62/-72 cells were cultured in the absence or presence of tetracycline for 10 days. (**C**) PRMT5 is required for cell proliferation. Growth rate of MCF7-pTR-7 and MCF7-PRMT5-KD-41/-62/-72 cells cultured in the absence or presence of tetracycline over a 8-day period. The average ± S.D. of triplicates was plotted, and the data is representative of three independent experiments. *P*-values were ≤0.005 (comparison upon induction for 8 days). (**D**) Deficiency in PRMT5 induces G1 arrest. MCF7-PRMT5-KD-41/-62/-72 cells were uninduced or induced to knockdown PRMT5 for 5 days. The percentage of cells in each phase of the cell cycle was quantified by FACS analysis. The average ± SEM of three independent experiments is shown. *P*-values were ≤0.01 (comparison of cells numbers in G1).

Thus, we generated MCF7 cell lines, MCF7-CARM1-PRMT1-KD-5/-9, in which shRNA targeting both CARM1 and PRMT1 are inducibly expressed (Figure S1A). To determine whether knockdown of both PRMTs has an effect on cell proliferation, we performed colony-formation assays (Figure S1B). We found that there was only a slight decrease in cell proliferation upon knockdown of CARM1 and PRMT1. However, the number of colonies was not significantly affected by deficiency in CARM1 and PRMT1 (Figure S1C).

To address whether CARM1 and PRMT1 knockdown has a short-term impact on cell proliferation, we performed a 9-day growth curve analysis (data not shown).

We found that knockdown of CARM1 and PRMT1 had no effect on the growth rate of MCF7 cells. Taken together, these data suggest that PRMT1 and CARM1 do not play an essential role in cell-cycle progression.

PRMT5 is required for both wild-type p53 and mutant p53 expression

To investigate whether PRMT5 modulates p53 levels and tumor suppressive function, MCF7-pTR-7 and MCF7-PRMT5-KD-41 were pretreated with tetracycline for 3 days, followed by treatment with doxorubicin, a DNA damage agent, for 6, 12 or 24 h. Western blot
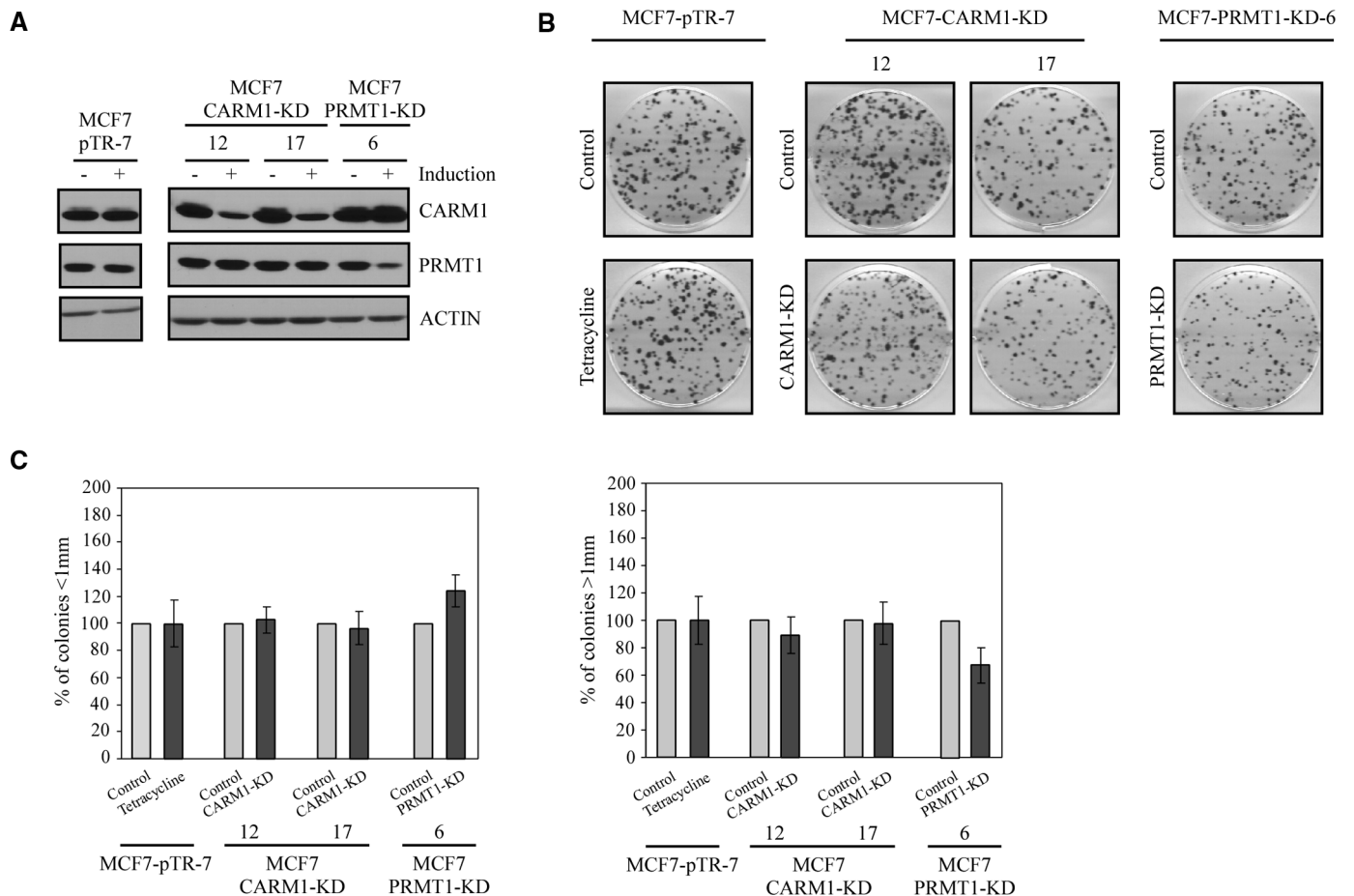
A

B

C

Figure 2. CARM1 and PRMT1 are not essential for cell proliferation. (**A**) Generation of MCF7 cell lines inducibly expressing CARM1 or PRMT1 shRNA. Levels of CARM1, PRMT1 and Actin were assayed in MCF7-pTR-7, MCF7-CARM1-KD-12/-17 and MCF7-PRMT1-KD-6 cells cultured in the absence (−) or presence (+) of tetracycline for 4 days. (**B**) CARM1 or PRMT1 are not required for colony formation. MCF7-pTR-7, MCF7-CARM1-KD-12/-17 and MCF7-PRMT1-KD-6 cells were cultured in the absence or presence of tetracycline for 10 days. (**C**) Quantification of the number of colonies shown in (B). The percentage of colonies with a diameter <1 mm (left panel) or >1 mm (right panel) was calculated in three representative areas for each cell line. The average ± SEM was plotted as the percentage of colonies increased or decreased by tetracycline compared to parental MCF7 cells.

analysis was performed to measure levels of PRMT5, MDM2, p53, p21 and GAPDH (Figure 3A). We showed that in MCF7-pTR-7 cells, doxorubicin stabilized endogenous p53 after 6 h treatment, which resulted in induction of p53 target genes, MDM2 and p21 (Figure 3A, compare lanes 1 and 3). A more pronounced effect on p53, MDM2 and p21 was detected after treatment with doxorubicin for 12 or 24 h (Figure 3A, compare lane 3 with lanes 5 and 7). In addition, we showed that tetracycline had no effect on p53 stabilization and induction of MDM2 or p21 upon doxorubicin treatment (Figure 3A, compare lanes 1, 3, 5 and 7 with lanes 2, 4, 6 and 8, respectively). As expected, a marked decrease in PRMT5 (by ≤45%) was detected in PRMT5 knockdown MCF7 cells upon induction of PRMT5 shRNA for 3 days (Figure 3A, compare lanes 9, 11, 13 and 15 with lanes 10, 12, 14 and 16, respectively). Interestingly, we also found that PRMT5 knockdown decreased (by 10%) p53 stabilization in the first 6 h, following treatment with doxorubicin (Figure 3A, compare lanes 11 and 12). In addition, a more pronounced effect on p53 stabilization was detected following treatment

with doxorubicin for 12 h (30% decrease) or 24 h (32% decrease) (Figure 3A, compare lanes 13 and 15 with lanes 14 and 16, respectively). Importantly, we showed that the decreased p53 stabilization upon PRMT5 knockdown led to a decrease in induction of MDM2 (by 40%) and p21 (by 60%) after 24 h doxorubicin treatment. Furthermore, we found that knockdown of PRMT5 had similar effects on p53 stabilization and its transcriptional activity in response to treatment with camptothecin, another DNA damage agent and Nutlin-3, a MDM2 inhibitor (data not shown). Taken together, our data suggest that arginine methyltransferase PRMT5 is required for efficient stabilization of p53 and its transcriptional activity in response to cellular stresses.

To confirm that the regulation of p53 upon PRMT5 knockdown is not cell-type specific, we examined whether PRMT5 is required for p53 stabilization in RKO and HCT116 colon carcinoma cell lines. These cell lines were transiently transfected with scrambled negative control siRNA or PRMT5 siRNA for 3 days, followed by treatment with doxorubicin for 12 and 24 h (Figure 3B).
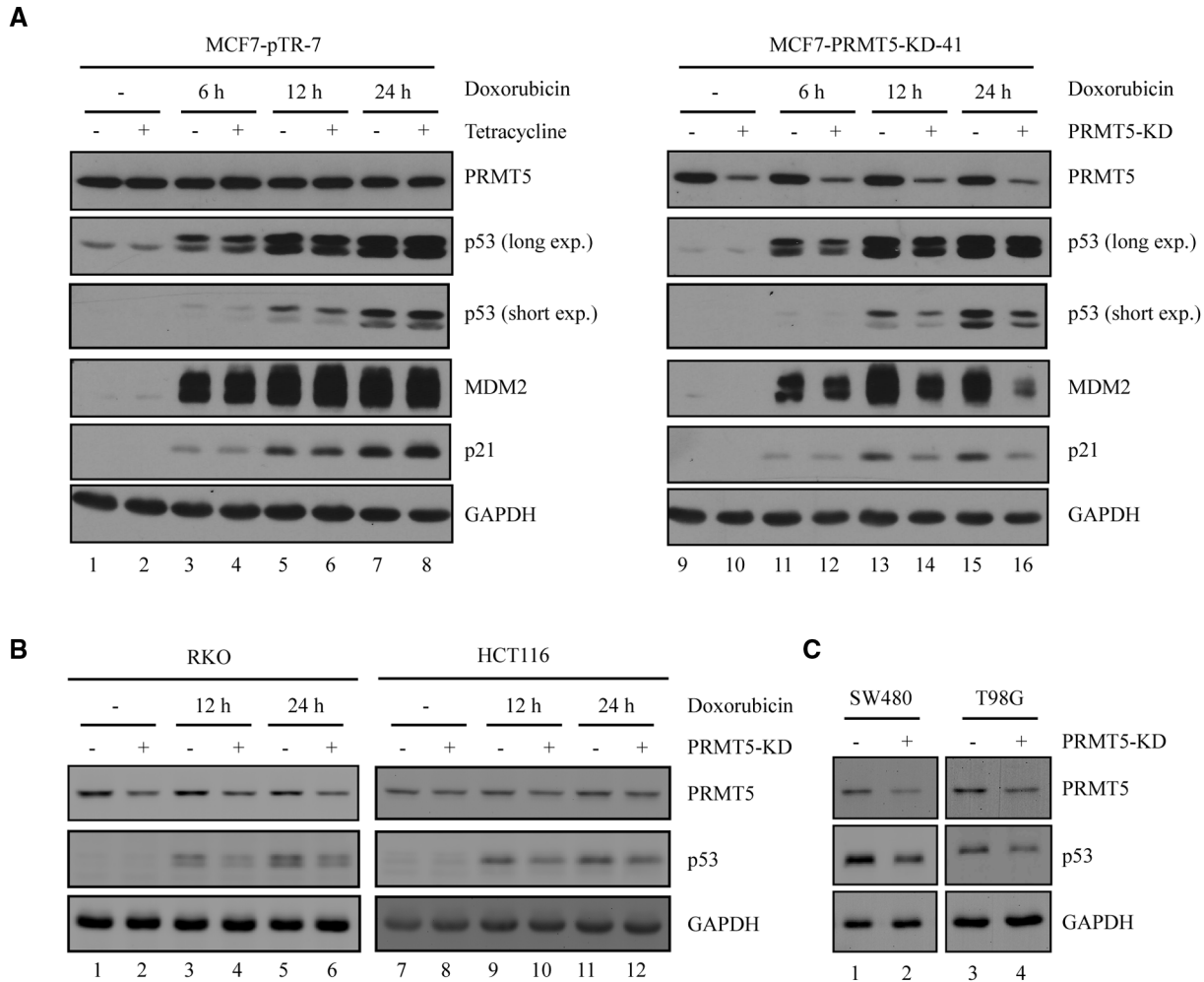
Figure 3. PRMT5 is required for efficient p53 stabilization and transcriptional activity in multiple cell lines. (**A**) Cells extracts were prepared from MCF7-pTR-7 and MCF7-PRMT5-KD-41 cells uninduced (−) or induced (+) to knockdown PRMT5 for 3 days, and then untreated or treated with 0.35 µM doxorubicin for 6, 12 or 24 h. (**B**) Cells extracts were prepared from RKO and HCT116 cells transiently transfected with scrambled control siRNA (−) or PRMT5 siRNA (+) for 3 days, and then untreated or treated with 0.35 µM doxorubicin for 12 or 24 h. (**C**) Cells extracts were prepared from SW480 and T98G cells transiently transfected with scrambled control siRNA (−) or PRMT5 siRNA (+) for 3 days. The data is representative of two independent experiments. Levels of PRMT5, p53, MDM2, p21 and GAPDH were detected by western blot analysis.

As expected, PRMT5 levels were decreased in RKO (by 40%) and HCT116 (by 25%) cells upon induction of PRMT5 siRNA for 3 days (Figure 3B, compare lanes 1 and 7 with lanes 2 and 8, respectively). Interestingly, we found that PRMT5 knockdown also decreased p53 stabilization in RKO (by 35%) and HCT116 (by 25%) cells following treatment with doxorubicin for 12 and 24 h (Figure 3B, compare lanes 3, 5, 9 and 11 with lanes 4, 6, 10 and 12, respectively). Together, these results suggest that PRMT5 is required for efficient stabilization of p53 in response to cellular stresses in multiple cell lines.

High expression of mutant p53 is frequently found in human tumors and contributes to tumorigenesis. To determine whether a deficiency in PRMT5 has an effect on mutant p53 expression, SW480 colon adenocarcinoma and T98G glioblastoma cell lines were treated with scrambled siRNA or PRMT5 siRNA for 3 days (Figure 3C). As expected, a decrease in PRMT5 was

detected in SW480 (by 34%) and T98G (by 28%) cells upon induction of PRMT5 siRNA (Figure 3C, compare lanes 1 and 3 with lanes 2 and 4, respectively). Interestingly, we found that PRMT5 knockdown decreased mutant p53 expression in SW480 (by 38%) and T98G (by 30%) cells. These data revealed that similarly to wild-type p53, mutant p53 expression is regulated by PRMT5.

To investigate whether CARM1 or PRMT1 regulates p53 stability and transcriptional activity, MCF7-CARM1-KD-12 and MCF7-PRMT1-KD-6 were pretreated with tetracycline for 3 days, followed by treatment with doxorubicin for 3, 6 or 12 h (Figure S2A). Here, we showed that CARM1 knockdown had little, if any, effect on p53 stabilization and MDM2 induction upon doxorubicin treatment (Figure S2A, compare lanes 3, 5 and 7 with lanes 4, 6 and 8). We also found that PRMT1 knockdown only slightly decreased p53
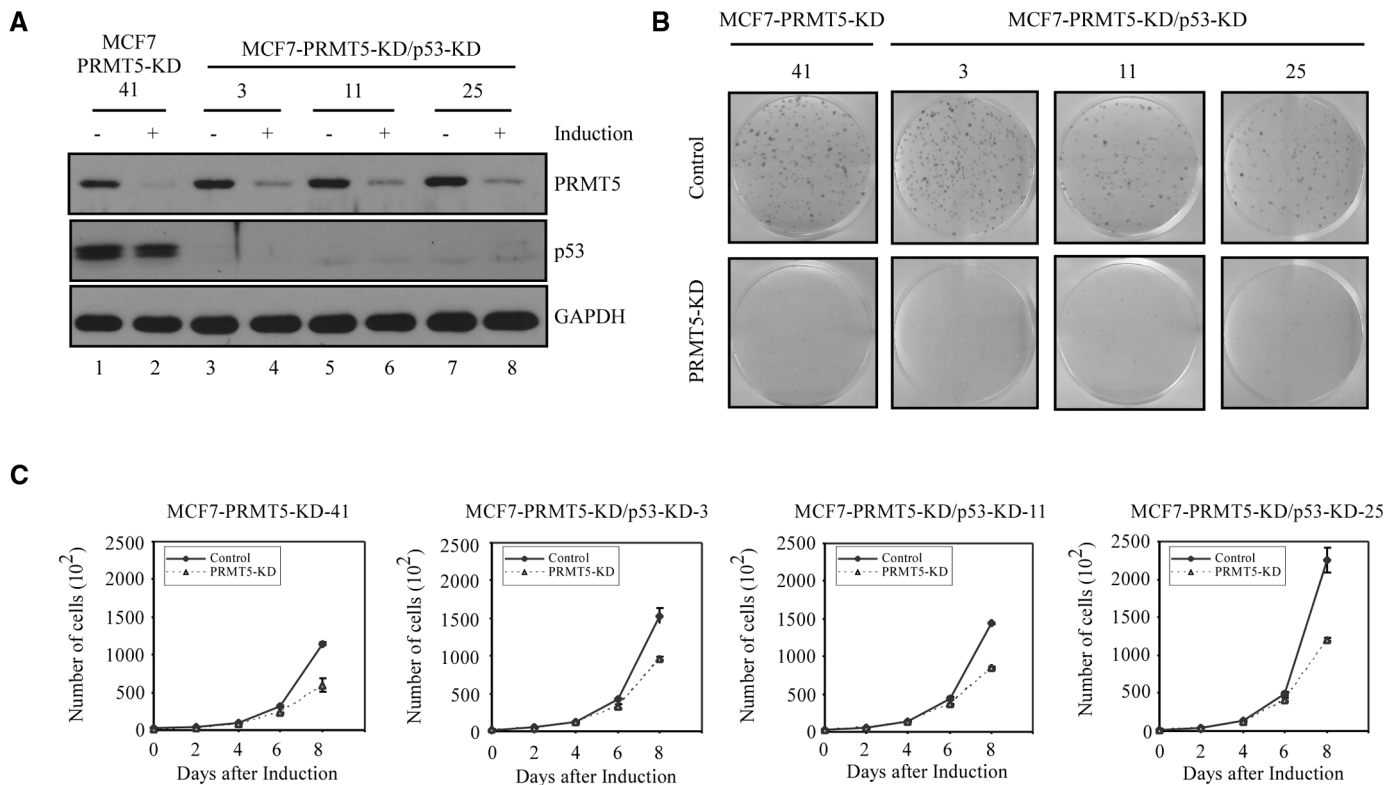
Figure 4. Knockdown of PRMT5 inhibits cell proliferation in a p53-independent manner. (**A**) Generation of MCF7 cell lines inducibly expressing PRMT5 shRNA and stably expressing p53 shRNA. Levels of PRMT5, p53 and GAPDH were assayed in MCF7-PRMT5-KD-41 and MCF7-PRMT5-KD/p53-KD-3/-11/-25 cells cultured in the absence (−) or presence (+) of tetracycline for 3 days. (**B**) PRMT5 is required for colony formation independently of p53. MCF7-PRMT5-KD-41 and MCF7-PRMT5-KD/p53-KD-3/-11/-25 cells were cultured in the absence or presence of tetracycline for 10 days. The data is representative of two separate experiments performed in triplicate wells. (**C**) PRMT5 is required for cell proliferation independently of p53. Growth rate of MCF7-PRMT5-KD-41 and MCF7-PRMT5-KD/p53-KD-3/-11/-25 cells cultured in the absence or presence of tetracycline over a 8-day period. The average ± S.D. of triplicates was plotted and the data is representative of two independent experiments. *P*-values were ≤0.01 (comparison upon induction for 8 days).

stabilization upon treatment with doxorubicin for 6 h (Figure S2A, compare lanes 13 and 14). However, similarly to CARM1 knockdown, PRMT1 knockdown had no effect on p53 stabilization and MDM2 induction upon doxorubicin treatment for 12 h (Figure S2A, compare lanes 15 and 16).

To address whether knockdown of both CARM1 and PRMT1 regulates p53, MCF7-CARM1-PRMT1-KD-5/-9 were pretreated with tetracycline for 3 days, followed by treatment with doxorubicin for 3, 6 or 12 h (Figure S2B). We found that knockdown of both CARM1 and PRMT1 had little if any effect on p53 stabilization and MDM2 induction upon doxorubicin treatment (Figure S2B, compare lanes 3, 5, 7, 11, 13 and 15 with lanes 4, 6, 8, 12, 14 and 16, respectively). Taken together, these results suggest that CARM1 and PRMT1 do not play a major role in p53 stability and transcriptional activity in response to DNA damage.

PRMT5 knockdown inhibits cell proliferation in a p53-independent manner

The tumor suppressor p53 is a major regulator of cell proliferation. Thus, we wanted to determine whether p53 plays a role in the inhibition of cell proliferation induced

upon PRMT5 knockdown. To do this, MCF7 cell lines were generated, in which p53 is stably knocked down and PRMT5 is inducibly knocked down. Three representative cell lines, MCF7-PRMT5-KD/p53-KD-3/-11/-25, along with MCF7-PRMT5-KD-41 cell line, are shown in Figure 4A. We found that in all three MCF7-PRMT5-KD/p53-KD cell lines, p53 levels were undetectable (Figure 4A, p53 panel, compare lanes 1–2 with lanes 3–8). As expected, a marked decrease (by ≤70%) in PRMT5 was detected upon tetracycline treatment for 3 days (Figure 4A, compare lanes 1, 3, 5 and 7 with lanes 2, 4, 6 and 8, respectively). In addition, we showed that in MCF7-PRMT5-KD-41 cell line, basal p53 levels were decreased (by 20%) upon PRMT5 knockdown (Figure 4A, compare lanes 1 and 2). Next, we performed a colony formation assay to determine the long-term effect of p53 knockdown on cell proliferation (Figure 4B). We found that p53 knockdown has no effect on the inhibition of cell proliferation induced upon PRMT5 knockdown in MCF7-PRMT5-KD/p53-KD cell lines. Next, we performed an 8-day growth curve on MCF7-PRMT5-KD-41 and MCF7-PRMT5-KD/p53-KD-3/-11/-25 (Figure 4C). We found that the decrease in number of proliferating cells upon PRMT5 knockdown was similar between MCF7-PRMT5-KD-41 (48% decrease) and

MCF7-PRMT5-KD/p53-KD-3/-11/-25 cell lines (<40% decrease). Taken together, these data suggest that PRMT5 knockdown inhibits cell proliferation in a p53-independent manner.

PRMT5 knockdown inhibits p53 protein synthesis

To determine the mechanism through which PRMT5 regulates p53, we sought to examine whether PRMT5 knockdown enhances p53 degradation by 26S proteasome. MCF7-PRMT5-KD-41 cells were uninduced or induced to knock down PRMT5 for 3 days, followed by treatment with MG132, a proteasome inhibitor, for 4 or 8 h (Figure 5A). As expected, PRMT5 was efficiently knocked down upon induction of PRMT5 shRNA for 3 days (Figure 5A, compare lane 1, 3 and 5 with lanes 2, 4 and 6, respectively). Interestingly, we showed that p53 was stabilized upon treatment with MG132 for 4 h (by 2.5-fold) and 8 h (by 3-fold) (Figure 5A, compare lanes 1 with lanes 3 and 5). Similarly, p53 levels were increased in PRMT5 knockdown cells treated with MG132 for 4 h (by 2.7-fold) and 8 h (by 2.9-fold) (Figure 5A, compare lanes 2 with lanes 4 and 6). However, we found that the knockdown of PRMT5 decreased p53 to a similar extent in the absence or presence of MG132 (Figure 5A, compare lanes 1, 3 and 5 with lanes 2, 4 and 6, respectively). To check whether PRMT5 knockdown regulates p53 degradation in response to DNA damage, MCF7-PRMT5-KD-41 cells uninduced or induced to knockdown PRMT5 for 3 days were treated with MG132 for 4 or 8 h, followed by doxorubicin treatment for 6 h (Figure 5B). Here, we found that PRMT5 knockdown prevented p53 stabilization upon treatment with doxorubicin, regardless of the presence of MG132 (Figure 5B, compare lanes 7, 9 and 11 with lanes 8, 10 and 12, respectively). Taken together, our data suggest that PRMT5 regulates p53 degradation independently of the 26S proteasome or through a mechanism upstream of its degradation.

Next, to assess whether PRMT5 regulates p53 protein half-life, MCF7-PRMT5-KD-41 cells were treated with cycloheximide, a protein synthesis inhibitor. As shown in Figure 5C, MCF7-PRMT5-KD-41 cells were pretreated with tetracycline for 3 days, followed by doxorubicin treatment for 6 h, and then treatment with cycloheximide for 30 min to up to 210 min. Western blot analysis was performed to measure levels of PRMT5, p53 and GAPDH (Figure 5C). As expected, we showed that in control and PRMT5 knockdown cells, p53 levels were similarly decreased upon cycloheximide treatment over the time course examined. For instance, we found that in control and PRMT5 knockdown cells, p53 levels were reduced by 35% upon treatment with cycloheximide for 90 min (Figure 5C, p53 panels, compare lanes 1 and 9 with lanes 4 and 12, respectively). Therefore, the half-life of p53 was similar in the presence or absence of PRMT5. Then, we wanted to determine whether changes in p53 protein levels upon PRMT5 knockdown were correlated with changes in *p53* transcript levels. To address this, real-time PCR experiments were performed on MCF7-PRMT5-KD-41 cells pretreated with tetracycline for 3 days, followed by doxorubicin treatment for 12 or

24 h (data not shown). We found that knockdown of PRMT5 had no effect on *p53* transcript levels in untreated cells and only slightly decreased *p53* transcript levels in cells treated with doxorubicin for 12 h. Therefore, our data suggest that PRMT5 regulates p53 downstream of its transcription.

To determine whether PRMT5 deficiency regulates p53 protein synthesis, we performed [^{35}S]methionine labeling on MCF7-PRMT5-KD-41 cells untreated or treated with tetracycline for 3 days, and p53 levels were determined by immunoprecipitation. Here, we found that knockdown of PRMT5 had no effect on overall [^{35}S]methionine-labeled proteins levels (Figure 5D, compare lanes 1 and 2). However, we showed that PRMT5 knockdown clearly decreased levels of newly synthesized p53 proteins (Figure 5D, compare lanes 5 and 6). Taken together, our data suggest that PRMT5 regulates p53 translation.

PRMT5 is required for translation initiation factor 4E expression

To further address the mechanism through which PRMT5 regulates p53, we investigated whether PRMT5 regulates the expression of eukaryotic translation initiation factor 4E (eIF4E). eIF4E is a key component in protein synthesis and therefore plays an important role in cell proliferation (26). eIF4E is also a mediator of Akt-mTOR signaling pathway to promote tumorigenesis (27). As shown in Figure 6A, MCF7-pTR-7 and MCF7-PRMT5-KD-41 cell lines were pretreated with tetracycline for 3 days, followed by doxorubicin treatment for 12 or 24 h. Western blot analysis was performed to measure levels of PRMT5, eIF4E and GAPDH (Figure 6A). We found that in MCF7-pTR-7 cells, tetracycline had no effect on eIF4E expression (Figure 6A, compare lanes 1, 3 and 5 with lanes 2, 4 and 6, respectively). In addition, treatment with doxorubicin had no effect on eIF4E expression (Figure 6A, compare lane 1 with lanes 3 and 5). In contrast, we found that in MCF7-PRMT5-KD-41, PRMT5 knockdown induced a marked decrease (by ≥40%) in eIF4E, regardless of the presence of doxorubicin (Figure 6A, compare lanes 7, 9 and 11, with lanes 8, 10 and 12, respectively). Taken together, these data suggest that PRMT5 has a role in protein translation through regulating eIF4E expression.

To further determine the significance of eIF4E regulation by PRMT5, we assessed whether eIF4E plays a role in the short-term regulation of cell proliferation by PRMT5. To do this, MCF7-PRMT5-KD-41 cells were transfected with pcDNA3 vector or pcDNA3 vector expressing Myc-tagged eIF4E for 24 h, followed by treatment with tetracycline for 3 days. Western blot analysis was performed to measure levels of PRMT5, Myc-tagged eIF4E and GAPDH (Figure 6B). As expected, a clear decrease in PRMT5 (by ≤45%) was detected in PRMT5 knockdown MCF7 cells upon induction of PRMT5 shRNA for 3 days (Figure 6B, compare lanes 1 and 3 with lanes 2 and 4, respectively). In addition, exogenous eIF4E expression was detected in cells transfected with pcDNA3 vector containing Myc-tagged eIF4E (Figure 6B, compare lanes 1 and 2 with lanes 3 and 4, respectively). Next, the number
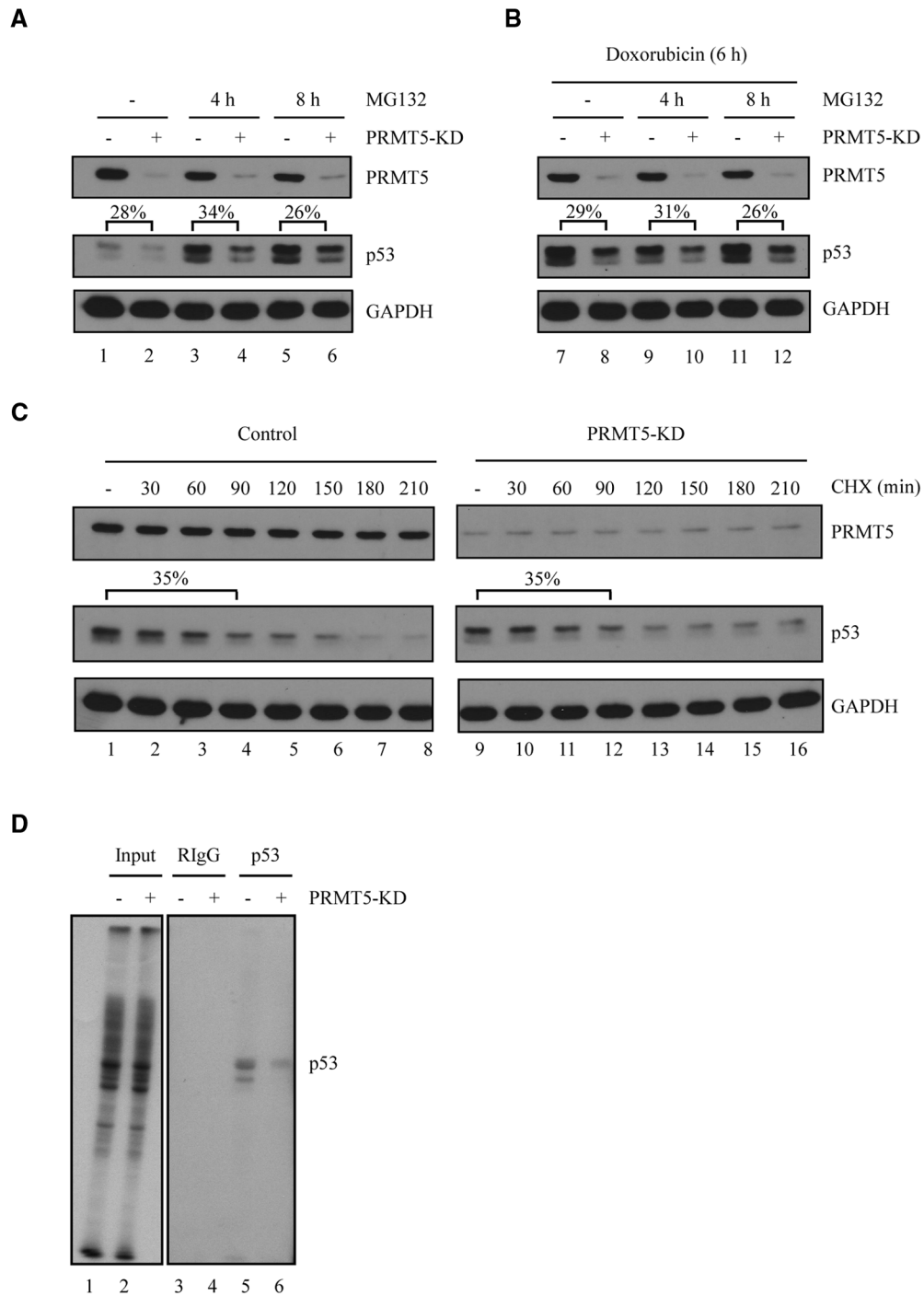
A

B

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D

Figure 5. Knockdown of PRMT5 inhibits p53 protein synthesis. (**A** and **B**) MCF7-PRMT5-KD-41 uninduced (−) or induced (+) to knockdown PRMT5 for 3 days were (A) untreated or treated with 10 μM MG132 for 4 or 8 h, (B) followed by treatment with doxorubicin for 6 h. Cells extracts were prepared to determine levels of PRMT5, p53, and GAPDH by western blot analysis. (**C**) MCF7-PRMT5-KD-41 cells were cultured in the absence or presence of tetracycline for 3 days and then treated with doxorubicin for 6 h, followed by treatment with cycloheximide (CHX) for the indicated times. Levels of PRMT5, p53 and GAPDH were assayed by western blot analysis. (**D**) PRMT5 knockdown inhibits p53 protein synthesis. MCF7-PRMT5-KD-41 cells were uninduced (−) or induced (+) to knockdown PRMT5 for 3 days and [^{35}S] methionine-labeled for 30 min. Whole-cell extracts and cells extracts immunoprecipitated with rabbit IgG or antibodies against p53 were resolved by SDS-PAGE and autoradiography. The data is representative of three independent immunoprecipitation experiments.

of proliferating cells was measured, and we found that knockdown of PRMT5 decreased MCF7 cell proliferation by 20% after 3 days (Figure 6C). Furthermore, we showed that eIF4E expression was sufficient to prevent short term

inhibition of cell proliferation by PRMT5 knockdown (Figure 6C). Taken together, these results revealed that the regulation of cell proliferation by PRMT5 is dependent on eIF4E.
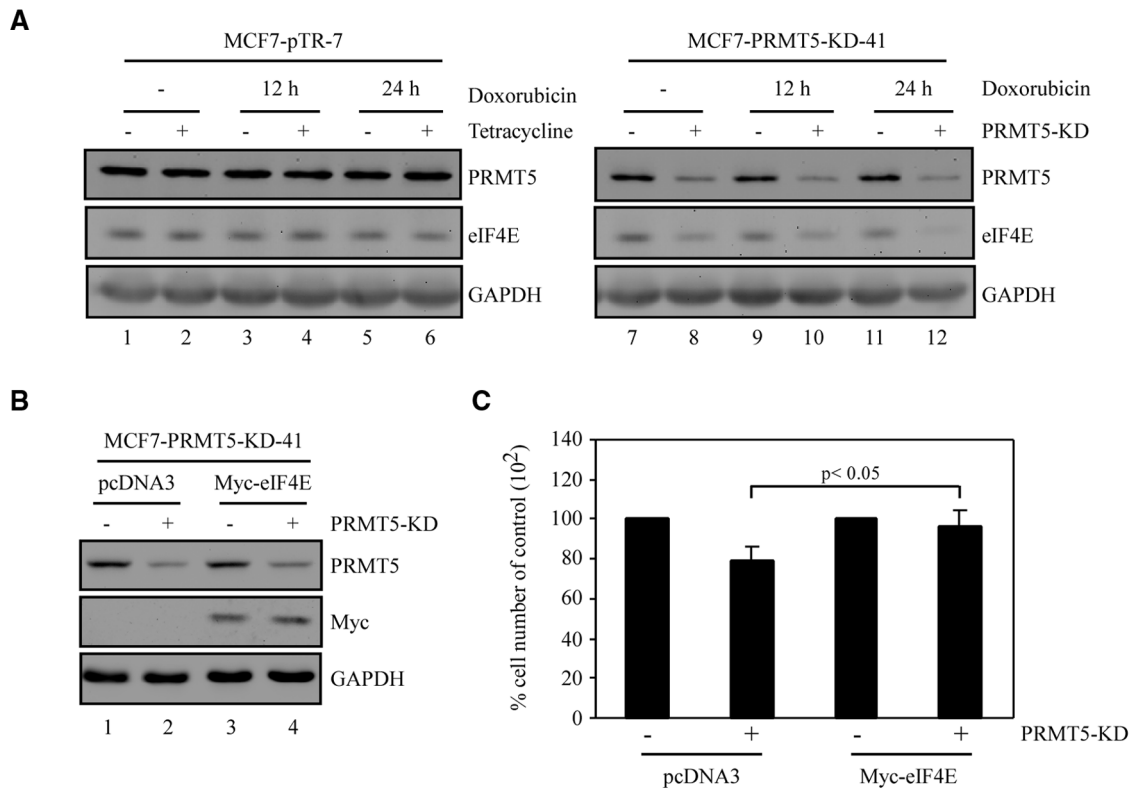
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Figure 6. PRMT5 knockdown inhibits the expression of translation initiation factor 4E. (**A**) Knockdown of PRMT5 inhibits eIF4E expression. Cells extracts were prepared from MCF7-pTR-7 and MCF7-PRMT5-KD-41 cells uninduced (−) or induced (+) to knockdown PRMT5 for 3 days, and then untreated or treated with 0.35 μM doxorubicin for 12 or 24 h. Levels of PRMT5, eIF4E and GAPDH were detected by western blot analysis. (**B**) MCF7-PRMT5-KD-41 cells were transfected with pcDNA3 or pcDNA3 vector expressing Myc-tagged eIF4E for 24 h, followed by treatment with tetracycline for 3 days. Levels of PRMT5, Myc-tagged eIF4E, and GAPDH were detected by western blot analysis. (**C**) eIF4E expression attenuates the inhibition of cell proliferation by PRMT5 knockdown. Cells were collected and counted by Coulter counter. The average ± SEM of triplicates was plotted as percentage of cells compared to untreated cells.

DISCUSSION

PRMTs have been implicated in many essential cellular processes ranging from gene expression to cell signaling. However, studies are needed to explore the physiological significance of many novel PRMTs identified in the last few years. In addition, there is little information on the potential interplay between class I and II PRMTs. Here, we found that class I and class II PRMTs have distinct roles in cell proliferation. Indeed, we found that two members of class I PRMTs, CARM1 and PRMT1, are minimally required for normal cell proliferation. Consistent with this, a recent report showed that CARM1 is specifically required for induction of cell-cycle progression by ERα in response to estrogen (28). It is therefore likely that similarly to CARM1, PRMT1 plays a role in stimulating cell proliferation in response to hormones or other signaling molecules.

In contrast to CARM1 and PRMT1, we showed here that PRMT5, a class II PRMT, is required for normal cell proliferation. Indeed, we found that PRMT5 regulates cell-cycle transition from G1 to S phase. PRMT5 is a well-known co-repressor present in chromatin-remodeling repressor complexes (17,18). Indeed, constitutive expression of PRMT5 antisense vector in NIH 3T3 cells was found to up-regulate numerous genes, including tumor

suppressor ST7 and cell-cycle regulator cyclin E2 (17). However, specific target genes involved in PRMT5-mediated cell proliferation still remain to be determined. Interestingly, an oncogenic role for PRMT5 has been supported by studies showing an increased PRMT5 level associated with gastric and lymphoid cancers (29,30).

Besides its role as transcriptional co-repressor, PRMT5 is involved in many other cellular processes. Indeed, PRMT5 facilitates myoD-induced muscle differentiation by mediating dimethylation of histone H3R8 and recruitment of Brg1-dependent chromatin remodeling enzymes for gene activation (31). PRMT5 is involved in androgen receptor-mediated transcriptional activation, in a manner independent of its methyltransferase activity (19). PRMT5 is also a component of the 20S methylosome and methylates Sm proteins, which are essential proteins for snRNPs biogenesis and RNA splicing (32,33). Recently, Jansson *et al.* (21) reported that p53 activity is regulated by PRMT5. The authors showed that PRMT5 methylates p53 at selected arginine residues in p53 oligomerization domain. In addition, PRMT5 deficiency was found to have multiple effects on p53, such as reduction in p53 levels, nuclear localization, and oligomerization activity. Consistent with this, we found that deficiency in PRMT5 reduces basal p53 levels and p53 transcriptional activity in

response to DNA damage. Furthermore, we revealed that PRMT5 regulates p53 protein synthesis. Studies have reported the regulation of p53 mRNA translation by ribosomal proteins, such as L26, and nucleolin although the mechanisms involved are still poorly understood (34–36). Here, we found that PRMT5 regulates the expression of eIF4E, a major component of the translation machinery involved in the mRNA-ribosome-binding step of protein synthesis. Indeed, eIF4E is a potent oncogene, which was previously shown to promote malignant transformation *in vitro* and human cancer formation (37,38). Indeed, cell proliferation mediated by PRMT5 was dependent on eIF4E, but independent of p53. We speculate that eIF4E is involved in regulating key growth-promoting factors at the translational level. However, in response to stress signals, PRMT5, likely through eIF4E regulation, plays an important role in providing sufficient p53 activation to induce pro-survival mechanisms, such as cell-cycle arrest and DNA repair, to ultimately limit the extent of damage to DNA. Taken together, our study revealed that the arginine methyltransferase PRMT5 is a pro-survival factor, which regulates cell proliferation in unstressed conditions and mediates efficient p53 response to protect against cellular stresses.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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