

# Low levels of miR-92b/96 induce PRMT5 translation and H3R8/H4R3 methylation in mantle cell lymphoma

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**Protein arginine methyltransferase PRMT5 interacts with human SWI/SNF complexes and methylates histones H3R8 and H4R3. To elucidate the role of PRMT5 in human cancer, we analyzed PRMT5 expression in normal human B lymphocytes and a panel of lymphoid cancer cell lines as well as mantle cell lymphoma (MCL) clinical samples. We show that PRMT5 protein levels are elevated in all cancer cells, including clinical samples examined despite its low rate of transcription and messenger RNA stability. Remarkably, polysome profiling revealed that PRMT5 mRNA is translated more efficiently in Mino and JeKo MCL cells than in normal B cells, and that decreased miR-92b and miR-96 expression augments PRMT5 translation. Consequently, global methylation of H3R8 and H4R3 is increased and is accompanied by repression of suppressor of tumorigenicity 7 (ST7) in lymphoid cancer cells. Furthermore, knockdown of PRMT5 expression reduces proliferation of transformed JeKo and Raji cells. Thus, our studies indicate that aberrant expression of PRMT5 leads to altered epigenetic modification of chromatin, which in turn impacts transcriptional performance of anti-cancer genes and growth of transformed lymphoid cells.**

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## Introduction

Human malignancies arise due to aberrant expression of genes that regulate cell growth and proliferation, and understanding the molecular mechanisms that contribute to altered gene expression is important for the design of therapies that specifically target affected central regulatory networks.

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Recent work has shown that chromatin-modifying enzymes, which can either activate or repress gene expression, play a crucial role in cancer etiology (Santos-Rosa and Caldas, 2005; Esteller, 2006). Chromatin can be altered by various mechanisms, including covalent modification of histones and/or DNA and non-covalent perturbation of histone–DNA contacts within nucleosomes. The net outcome of these chromatin alterations is to modulate accessibility to both repressor and activator proteins (Sif, 2004; Martin and Zhang, 2005). Several reports have shown that the interplay and crosstalk between chromatin-modifying enzymes is necessary for efficient regulation of gene expression, and that changes that affect the activity or targeting of chromatin remodelers can trigger cancer (Fischle *et al*, 2003; Hake *et al*, 2004).

Histone modifications like acetylation, methylation and ubiquitination have emerged as important marks in the control of gene expression, which can act either synergistically or antagonistically to specify transcriptional performance (Fischle *et al*, 2003). Both histone lysine and arginine residues can be methylated by SET proteins and protein arginine methyltransferases (PRMT), respectively, and studies by different groups have shown that methylation can either inhibit or induce transcription depending on the type and residue being modified (Bedford and Richard, 2005; Martin and Zhang, 2005). For instance, trimethylation of H3K4 is associated with active transcription and acts synergistically with H3 and H4 acetylation to activate *Hoxc8* expression (Milne *et al*, 2002). In contrast, trimethylation of H3K9 and/or H3K27 induces gene silencing and appears to be excluded from *Ubx* promoter regions that harbor trimethylated H3K4 (Cao *et al*, 2002; Hall *et al*, 2002; Papp and Muller, 2006).

PRMTs are evolutionarily conserved and PRMT5 is one of the type II arginine methyltransferases that catalyze  $\omega$ -N<sup>G</sup>-monomethylation and  $\omega$ -N<sup>G</sup>,N<sup>G</sup>-symmetric dimethylation (Bedford and Richard, 2005). PRMT5 interacts with a number of multisubunit complexes and is involved in a variety of cellular processes, including RNA processing, signal transduction, transcriptional regulation and germ cell development (Pollack *et al*, 1999; Friesen *et al*, 2001; Fabbri *et al*, 2002; Pal *et al*, 2003, 2004; Ancelin *et al*, 2006). Interestingly, PRMT5 can impact transcription in either a methylase-dependent or -independent manner. As a component of the androgen receptor cofactor complex, PRMT5 positively modulates androgen receptor-driven transcription independent of its methyltransferase activity (Hosohata *et al*, 2003); however, when PRMT5 is associated with hSWI/SNF complexes, its chromatin-modifying activity is important for histones H3 and H4 methylation, and regulation of transcription (Pal *et al*, 2004; Dacwag *et al*, 2007). Furthermore, methylation by PRMT5 can also have either a positive or negative effect on its substrates. For example, PRMT5-

mediated methylation of SmD1 and SmD3 is crucial for their incorporation into snRNPs, which are involved in RNA splicing, whereas methylation of the MBD2 subunit of NURD reduces its ability to associate with methylated DNA and to repress transcription (Friesen *et al*, 2001; Guezennec *et al*, 2006; Tan and Nakielny, 2006).

Recent work indicates that PRMT5 can modulate gene expression either directly by modifying histones or indirectly by altering the activity of various transcription factors (Fabbri *et al*, 2002; Kwak *et al*, 2003; Pal *et al*, 2003, 2004; Guezennec *et al*, 2006; Tan and Nakielny, 2006). Direct evidence for the involvement of PRMT5 in transcriptional regulation comes from studies that clearly demonstrate that PRMT5 interacts with chromatin remodeling complexes such as BRG1/BRM-based hSWI/SNF complexes and MBD2-based NURD complexes (Pal *et al*, 2003, 2004; Guezennec *et al*, 2006). As part of these chromatin remodelers, PRMT5 is able to methylate histones H3 and H4 and repress transcription of tumor suppressor genes such as suppressor of tumorigenicity 7 (*ST7*) and *NM23-H1*, and cell cycle regulators such as *CYCLIN E1*, *P14<sup>ARF</sup>* and *P16<sup>INK4a</sup>*.

To assess the role of PRMT5 in human lymphoid cancers, we analyzed its expression in a panel of transformed lymphoid cells including mantle cell lymphoma (MCL) cell lines and MCL patient samples. MCL is a subtype of non-Hodgkin's B cell lymphoma characterized by the presence of t(11;14) (q13;q32) chromosomal translocation. Patients afflicted by MCL show a very poor prognosis and survival rate of 3–4 years. MCL is incurable due to the lack of effective treatments, and identification of novel therapeutic targets remains the focus of ongoing MCL research (Witzig, 2005). Using patient-derived MCL cell lines as a model system, we examined the level of PRMT5 and global methylation of histones H3R8 and H4R3, which are preferentially symmetrically methylated by PRMT5. We found that PRMT5 is highly expressed in a wide variety of lymphoid cancer cell lines, including MCL clinical samples, and consequently global symmetric methylation of H3R8 and H4R3 is also altered in transformed lymphoid cell lines and MCL clinical samples. We also show that increased PRMT5 protein expression is regulated at the translational level and correlates with transcriptional silencing of *ST7*. Furthermore, knockdown of PRMT5 expression interferes with growth of transformed B cells. Thus, our work identifies PRMT5 as a key chromatin regulator whose aberrant expression affects global H3R8 and H4R3 methylation, contributes to *ST7* tumor suppressor gene silencing and is associated with lymphomagenesis.

## Results

### ***PRMT5 is overexpressed in lymphoid cancer cell lines and its level correlates with increased symmetric methylation of histones H3R8 and H4R3***

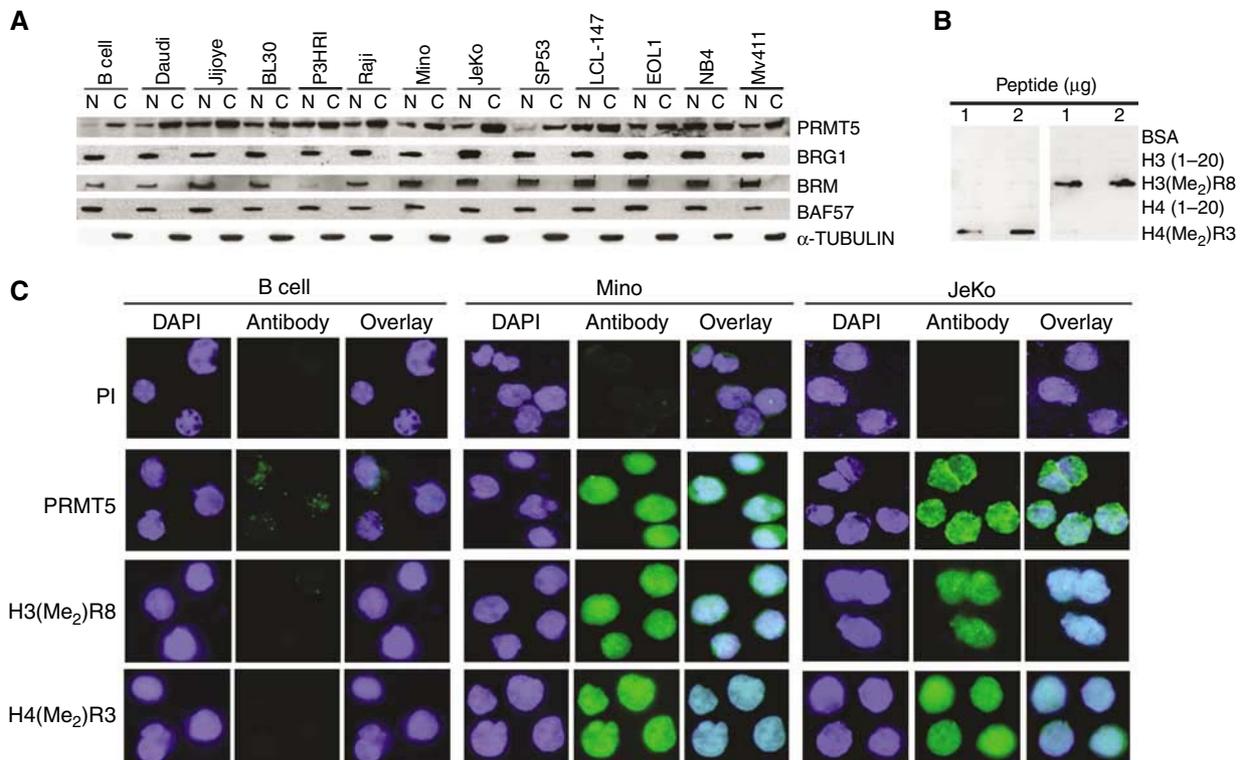
We have previously shown that PRMT5 can interact with BRG1 and BRM-based hSWI/SNF, and that overexpression of PRMT5 in an immortalized, but not transformed cell line, induces hyperproliferation and cellular transformation (Pal *et al*, 2004). This latter result prompted us to investigate the role of PRMT5 in human malignancies of lymphoid origin for which normal controls are readily available. Nuclear

and cytosolic extracts from normal CD19<sup>+</sup> B lymphocytes, patient-derived lymphoma (Burkitt's lymphoma: Daudi, Jijoye, BL30, P3HRI, and Raji; MCL: Mino, JeKo and SP53), *in vitro* EBV-transformed lymphoma (LCL-147) and leukemia cell lines (EOL1, NB4, Mv411) were analyzed by Western blotting (Figure 1A). PRMT5 protein levels were elevated in both nuclear and cytosolic fractions of transformed lymphoid cells. As PRMT5 associates with BRG1 and BRM chromatin remodeling complexes and mutations of hSWI/SNF components have been implicated in various human cancers including leukemia and lymphoma, we analyzed the levels of BRG1, BRM, and BAF57 in normal and transformed lymphoid cells. hSWI/SNF subunits were expressed in all samples except for the Burkitt's lymphoma P3HRI cell line where BRM expression was reduced. Because PRMT5 was found in both nuclear and cytosolic fractions, we measured expression of  $\alpha$ -TUBULIN and found that it was expressed exclusively in the cytosol, indicating that the presence of PRMT5 in the nucleus is not due to cytosolic contamination.

To further confirm the Western blot data, we stained normal B and patient-derived Mino and JeKo MCL cells with either pre-immune or immune anti-PRMT5 antibody (Figure 1C). A signal was detected only when immune anti-PRMT5 antibody was used, which was significantly higher in both Mino and JeKo MCL cell lines, suggesting that the level of PRMT5 protein is elevated in transformed lymphoid cells. We have previously shown that PRMT5 can methylate histones H3R8 and H4R3 *in vitro*, and we have linked PRMT5 recruitment to symmetric methylation of H3R8 *in vivo* (Pal *et al*, 2004). Therefore, we analyzed global H3R8 and H4R3 methylation in normal B and MCL cells using antibodies that can specifically recognize either symmetrically methylated H3R8 or H4R3 (Figure 1B, and Supplementary Figure 1A and B). Further characterization of both antibodies using whole-cell extracts revealed that the anti-H3(Me<sub>2</sub>)R8 antibody is highly specific, whereas the anti-H4(Me<sub>2</sub>)R3 antibody weakly crossreacts with four other polypeptides in addition to symmetrically methylated H4R3 (Supplementary Figure 1C and E). When anti-H3(Me<sub>2</sub>)R8 and anti-H4(Me<sub>2</sub>)R3 antibodies were used in immunofluorescence studies, they failed to detect these epigenetic marks in normal B lymphocytes; however, Mino and JeKo MCL cell lines exhibited high levels of symmetrically methylated H3R8 and H4R3 (Figure 1C). Similar results were observed when core histones isolated from normal and transformed B cells were analyzed by Western blotting using anti-H3(Me<sub>2</sub>)R8 and anti-H4(Me<sub>2</sub>)R3 antibodies (Supplementary Figure 1D and E). Collectively, these studies suggest that increased expression of PRMT5 induces symmetric methylation of H3R8 and H4R3, and is associated with MCL pathology.

### ***PRMT5 mRNA expression and stability are altered in patient-derived Mino and JeKo MCL cell lines***

Having found that expression of PRMT5 protein is enhanced in various transformed lymphoid cell lines, we wanted to determine whether this was a direct result of increased *PRMT5* mRNA expression (Figure 2A). Real-time RT-PCR revealed that, despite elevated levels of PRMT5 protein in cancer cells, the steady-state levels of *PRMT5* mRNA were 2- to 5-fold lower in transformed lymphoid cells ( $P < 10^{-4}$ ), suggesting that there are other mechanisms involved in



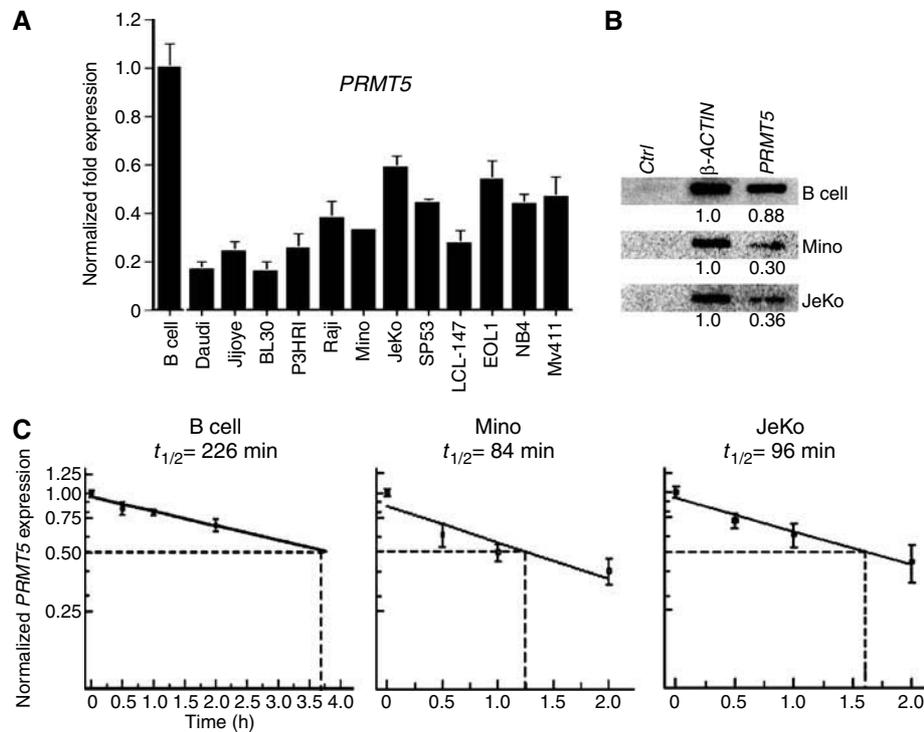
**Figure 1** PRMT5 is overexpressed in lymphoma and leukemia cell lines. (A) Expression of PRMT5 and hSWI/SNF subunits were assessed by Western blotting using either nuclear (N) and cytosolic (C) extracts from normal CD19<sup>+</sup> B cells, or the indicated transformed cell lines (20 μg). To discern between N and C fractions, we measured α-TUBULIN expression. (B) Anti-H3(Me<sub>2</sub>)R8 and anti-H4(Me<sub>2</sub>)R3 antibodies do not crossreact and are highly specific. Portions (1 and 2 μg) of BSA or the indicated peptides were spotted on nitrocellulose membrane, and detected using anti-H4(Me<sub>2</sub>)R3 or anti-H3(Me<sub>2</sub>)R8 antibodies. (C) Immunofluorescence of PRMT5, H4(Me<sub>2</sub>)R3, and H3(Me<sub>2</sub>)R8 in normal B cells, Mino, and JeKo cells. Normal B and MCL cells were fixed and incubated with either pre-immune, or immune anti-PRMT5, anti-H3(Me<sub>2</sub>)R8, and H4(Me<sub>2</sub>)R3 antibodies. FITC-labeled goat anti-rabbit antibody was used to detect PRMT5 and modified histones, and DAPI was used to stain nuclei. Pictures were taken at ×100 magnification.

upregulation of PRMT5 protein expression. To understand the underlying cause for decreased *PRMT5* mRNA expression in transformed lymphoid cells, we examined the rate of *PRMT5* transcription and mRNA stability in normal B cells as well as patient-derived Mino and JeKo MCL cell lines (Figures 2B and C). Consistent with the real-time RT-PCR results, nuclear run on analysis indicated that the rate of *PRMT5* mRNA transcription was 2.9- and 2.4-fold lower in Mino ( $P = 0.028$ ) and JeKo ( $P = 0.011$ ) MCL cell lines, respectively, suggesting that *PRMT5* mRNA transcription is more efficient in normal B lymphocytes. To measure *PRMT5* mRNA stability, normal B lymphocytes as well as Mino and JeKo MCL cell lines were treated with DRB to inhibit mRNA synthesis, and *PRMT5* mRNA levels were measured at various times by real-time RT-PCR. *18S* rRNA was used as an internal control to normalize *PRMT5* mRNA levels in these experiments, because low dose and short-time treatment with DRB does not affect RNA polymerase I transcription. We found that, in comparison to normal B cells ( $t_{1/2} = 226$  min), the half-life of *PRMT5* mRNA was reduced 2.7-fold in Mino ( $t_{1/2} = 84$  min) and 2.3-fold in JeKo ( $t_{1/2} = 96$  min) MCL cell lines ( $P = 0.009$ ). Taken together, these findings along with the observed decrease in *PRMT5* mRNA transcription suggest that the observed augmentation in PRMT5 steady-state levels could be the result of either increased *PRMT5* mRNA translation and/or enhanced PRMT5 protein stability in transformed MCL cell lines.

#### Aberrant expression of miR-92b and miR-96 is associated with enhanced PRMT5 translation

To analyze the efficiency of *PRMT5* mRNA translation, we performed sucrose gradient sedimentation experiments in the presence of cycloheximide using cell lysates from normal B lymphocytes and MCL cell lines (Figure 3A). Gradient fractions were collected, and total RNA was extracted and used to measure the levels of *PRMT5* mRNA by real-time RT-PCR. The amount of β-ACTIN mRNA in each fraction was used as an internal control to normalize the amount of *PRMT5* mRNA present in each fraction. Sucrose gradient analysis demonstrated that, although *PRMT5* mRNA was detected in polyribosomal fractions 11–18 in normal and transformed B cells, only 24% of total *PRMT5* mRNA was polyribosomal in normal B lymphocytes. However, the fraction of *PRMT5* mRNA associated with polyribosomes was enriched 2.2- and 2.6-fold in Mino ( $P < 10^{-4}$ ) and JeKo ( $P = 0.0009$ ) cells, respectively. This change in the polyribosome profile of *PRMT5* mRNA and the Western blot results suggest that *PRMT5* mRNA translation is enhanced in transformed Mino and JeKo MCL cell lines.

To elucidate the mechanism responsible for increased translation of *PRMT5*, we reasoned that there might be *PRMT5*-specific miRNAs involved in regulating its translation. We searched the Wellcome Trust Sanger Institute miRNA registry (<http://microrna.sanger.ac.uk>) for the presence of potential miRNA binding site(s) in the 3' untranslated



**Figure 2** Expression of *PRMT5* is regulated at different levels. (A) *PRMT5* mRNA expression was measured by real-time RT-PCR in normal as well as transformed B cells. The bar graph shows normalized fold expression of *PRMT5* mRNA in various cell lines relative to normal B cells using *GAPDH* as internal control. RT-PCR analysis was performed three times in triplicates and the graph shows the average  $\pm$  s.d. (B) The rate of *PRMT5* transcription is altered in MCL cell lines. Nuclear run on assays were conducted as described in materials and methods. Control (Ctrl) *PvuII-PvuII* DNA fragment of pBluescript KS(+), and  $\beta$ -ACTIN and *PRMT5* cDNA PCR fragments were immobilized on Hybond XL membrane and detected with radiolabeled RNA isolated from the indicated cells. Signals were quantitated and expression of *PRMT5* was reported relative to  $\beta$ -ACTIN. (C) *PRMT5* mRNA is more stable in B cells. Normal and transformed B cells were treated with DRB for the indicated times, and total RNA was isolated and quantitated by real-time RT-PCR. *PRMT5* mRNA expression was normalized using *18S* as internal control. To calculate the half-life ( $t_{1/2}$ ) of *PRMT5* mRNA, data points in each graph were generated from four distinct experiments conducted in triplicates.

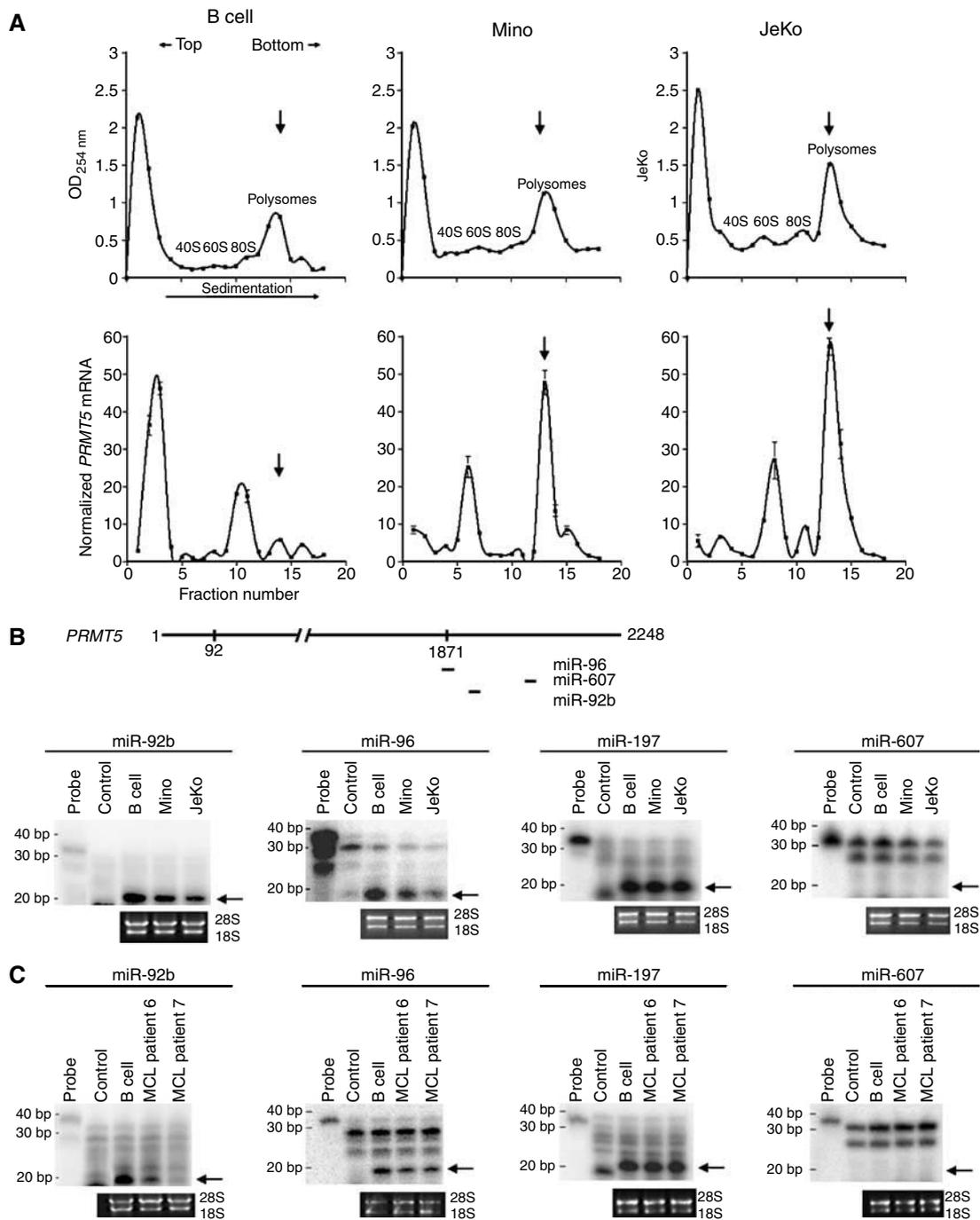
lated (UTR) region of *PRMT5*. We analyzed expression of 11 potential *PRMT5* targeting miRNAs in normal and transformed B cells using RNase protection assays (Figures 3B and C and data not shown). Of all the tested miRNAs, only miR-92b and miR-96 expression was detected; however, their expression was 2- to 2.5-fold lower in Mino (miR-92b  $P=0.009$ , miR-96  $P=0.012$ ) and JeKo (miR-92b  $P=0.01$ , miR-96  $P=0.005$ ) MCL cell lines, respectively (Figure 3B). When we analyzed the levels of an unrelated control miRNA, miR-197, we found that its expression was unaltered in normal and transformed B cells. More importantly, we also found reduced expression of miR-92b and miR-96 in MCL clinical samples 6 and 7, suggesting that aberrant expression of both miRNAs is a hallmark of MCL (Figure 3C).

**Re-expression of miR-92b and miR-96 inhibits PRMT5 translation in vivo, and their binding sites are critical for translational regulation**

To evaluate further the effect of miR-92b and miR-96 on *PRMT5* translational regulation *in vivo*, we electroporated JeKo cells with either wild-type or mutant miR-92b and miR-96 individually and checked for the levels of *PRMT5* protein (Figure 4A). In the presence of wild-type miR-92b, *PRMT5* protein expression was reduced by 80%, whereas miR-96 caused a 50% decrease in protein expression. Similarly, when we conducted the same experiment in Raji cells, *PRMT5* protein levels were reduced by 60–85%, highlighting the importance of miR-92b and miR-96 expression in transla-

tional regulation of *PRMT5* (Figure 4A). To verify whether reduced expression of *PRMT5* is the result of translational inhibition, but not *PRMT5* mRNA degradation, we measured *PRMT5* mRNA steady-state levels in mock-transfected cells as well as cells transfected with wild-type and mutant miR-92b or miR-96 (Supplementary Figure 2A and B). As expected, *PRMT5* mRNA levels were not significantly altered in the presence of exogenously transfected miR-92b or miR-96 in JeKo and Raji cell lines. As we have previously identified *ST7* as a *PRMT5* target gene, we analyzed its mRNA levels in miR-92b- or miR-96-electroporated Raji cells (Supplementary Figure 2C). In the presence of miR-92b, *ST7* mRNA levels were derepressed 2.2-fold ( $P=0.001$ ), whereas electroporation of miR-96 resulted in a 1.9-fold *ST7* derepression ( $P=0.005$ ). Taken together, these results suggest that decreased expression of *PRMT5* has a direct impact on *ST7* transcription.

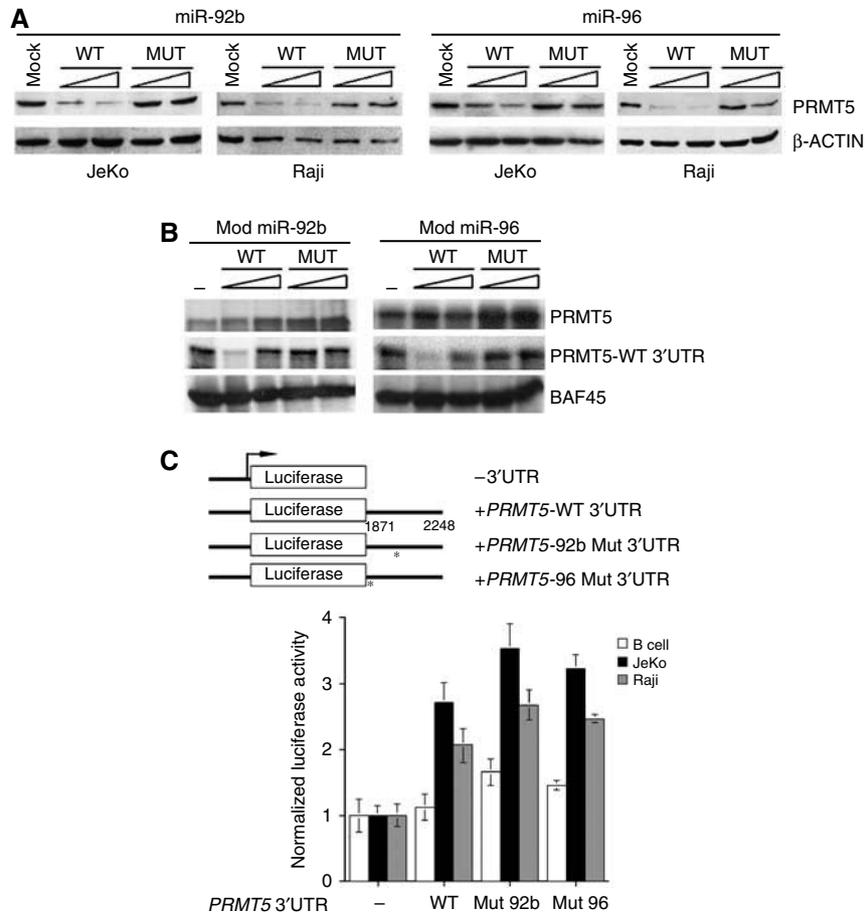
Next, we reconstituted miR-92b- and miR-96-mediated translational inhibition of *PRMT5 in vitro*. Recent work has shown that translational gene silencing by miRNAs *in vitro* requires the presence of a 7-methyl G cap and a poly(A) tail (Wang et al, 2006). Therefore, we conducted *in vitro* translation experiments using 7-methyl G-capped and poly(A)-tailed *PRMT5* mRNA with and without wild-type 3'UTR. Both wild-type and mutant miR-96 failed to inhibit *PRMT5* translation *in vitro*, suggesting that there are other components missing in the rabbit reticulocyte lysate, which might stabilize miR-96 binding to its target site (data not shown). Because miR-96



**Figure 3** *PRMT5* mRNA is translated more efficiently in MCL cell lines. (A) Polyribosome profiles of normal and transformed B cells. Whole-cell lysate were fractionated by 15–40% sucrose gradient sedimentation, and polyribosome profiles were determined by measuring the absorbance of each fraction at 254 nm (upper panel). Fractions representing 40S, 60S, 80S and polyribosomes are indicated. RNA isolated from each fraction was used to measure the level of *PRMT5* mRNA by real-time RT-PCR, and  $\beta$ -*ACTIN* was used as an internal control to normalize *PRMT5* mRNA levels in each fraction (lower panel). The amount of *PRMT5* mRNA present in each fraction is reported relative to the fraction containing the lowest copy number of *PRMT5* mRNA. The data points in each graph represent the average from triplicate RT-PCR reactions  $\pm$  s.d. Vertical arrows indicate peak polyribosome fractions. (B, C) Differential expression of *PRMT5*-specific miRNAs in normal and transformed B cells. Schematic representation of *PRMT5* mRNA depicting the position of potential miRNA-binding sites within the 3'UTR (B, upper panel). RPA was performed on 20  $\mu$ g (B) or 5  $\mu$ g (C) of total RNA isolated from the indicated cells using miR-92b, miR-96, miR-197, or miR-607 probe. Probe represents 1/10th of the total amount of labeled probe used in each reaction, and control shows digestion of the probe in presence of yeast tRNA. Ethidium bromide-stained gels are included to show equal loading. Arrows show position of mature miRNAs.

anneals to the *PRMT5* 3'UTR primarily through its seed sequence (+2 to +8), we assumed that the *PRMT5* mRNA:miR-96 hybrid molecule would not be stable thermodynamically at 30°C ( $\Delta G = -13.76$  kcal/mol,  $T_m = 22^\circ\text{C}$ ). To make up for the lack of stability, we changed six consecutive

ribonucleotides (+9 to +14) in miR-96 ( $\Delta G = -27.5$  kcal/mol,  $T_m = 44^\circ\text{C}$ ), and tested its ability to inhibit *PRMT5* translation *in vitro* (Figure 4B). Modified wild-type, but not mutant miR-96, where the seed sequence (+2 to +8) has been mutated, inhibited *PRMT5* translation efficiently at a 1:1



**Figure 4** PRMT5 expression is downregulated by miR-92b and miR-96 in transformed B cells. **(A)** JeKo and Raji cells were electroporated with 2.5 and 5.0  $\mu$ g of either wild-type or mutant miR-92b and miR-96 double-stranded RNA individually, and 20  $\mu$ g of RIPA extracts were analyzed by Western blotting. **(B)** Effect of modified miR-92b and miR-96 on PRMT5 expression *in vitro*. *In vitro* translation was carried out in the absence or presence of increasing amounts of modified wild-type or mutant miR-92b or miR-96 using 0.25  $\mu$ g of PRMT5 mRNA without 3'UTR (PRMT5) and with wild-type 3'UTR (PRMT5-WT 3'UTR). BAF45 mRNA was used as a control. **(C)** Normal B ( $25 \times 10^6$ ) or transformed JeKo and Raji ( $5 \times 10^6$ ) cells were electroporated in the presence of pRL-TK with either pCMV-LUC, pCMV-LUC fused to wild-type PRMT5 3'UTR, or pCMV-LUC fused to mutated miR-92b or miR-96-binding site PRMT5 3'UTR construct, and luciferase expression was measured using dual luciferase reporter assay. Luciferase activity is represented relative to pCMV-LUC for each cell line, and has been normalized using Renilla luciferase.

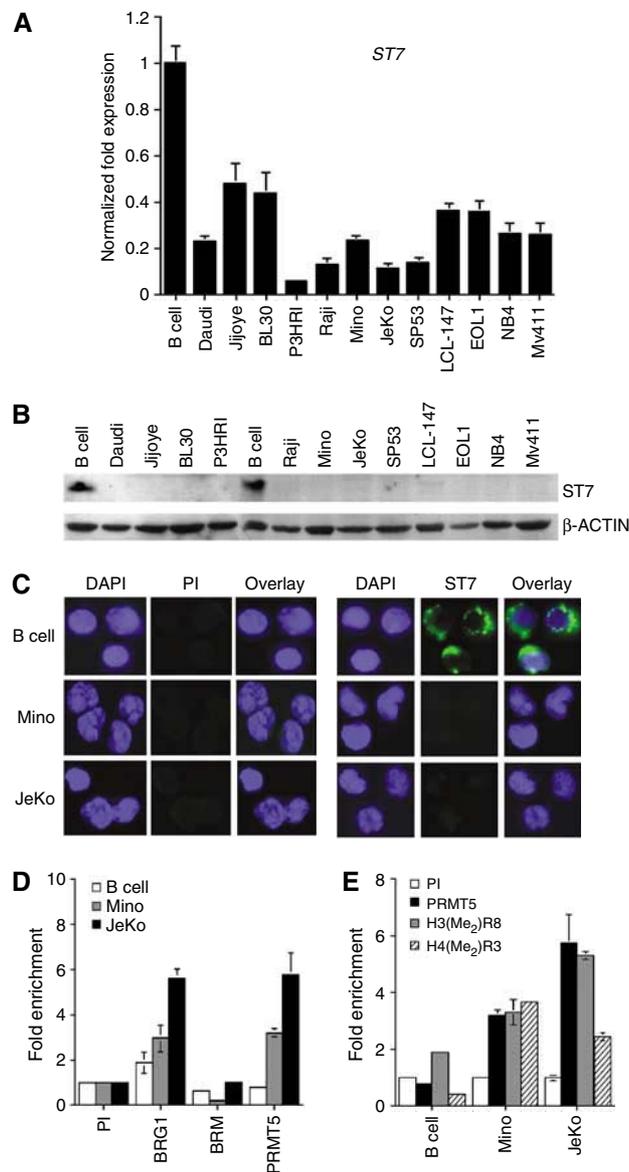
molar ratio when PRMT5 mRNA with wild-type 3'UTR was used. Translation of PRMT5 mRNA without 3'UTR and the unrelated BAF45 mRNA was unaffected. Similar results were observed when modified wild-type and mutant miR-92b were used in these *in vitro* translation experiments (Figure 4B).

In light of the findings, which showed that re-expression of either miR-92b or miR-96 reduces PRMT5 expression *in vivo*, we wanted to determine whether the binding sites of miR-92b and miR-96 were critical for translational regulation (Figure 4C). Wild-type and mutant PRMT5 3'UTRs were subcloned downstream of a CMV-driven luciferase reporter, and electroporated into either normal B lymphocytes or transformed JeKo and Raji cell lines. In the presence of wild-type PRMT5 3'UTR, luciferase expression was unchanged in B cells, whereas in JeKo and Raji cells it was enhanced 2.7- ( $P=0.004$ ) and 2-fold ( $P=0.026$ ), respectively, indicating that reduced expression of miR-92b and miR-96 in transformed lymphoid cells permits enhanced luciferase expression. When the miR-92b or miR-96 seed sequence binding site was mutated, luciferase expression was increased 1.5- ( $P=0.035$ ) to 1.7-fold ( $P=0.001$ ) in

B cells, 3.2- ( $P=0.006$ ) to 3.5-fold ( $P=0.003$ ) in JeKo cells, and 2.5- ( $P=0.037$ ) to 2.7-fold ( $P=0.008$ ) in Raji cells. These results show that miR-92b and miR-96 contribute to proper regulation of PRMT5 translation.

#### The PRMT5 target gene *ST7* is repressed in lymphoid cancer cell lines

We have previously identified *ST7* as a direct target gene of PRMT5, and demonstrated using a flag-tagged PRMT5 cell line that recruitment of PRMT5 to the *ST7* promoter induces symmetric methylation of histone H3R8 and is associated with *ST7* transcriptional repression (Pal *et al*, 2004). As PRMT5 protein levels are abnormally high in an array of transformed lymphoid cell lines, we investigated whether elevated levels of PRMT5 could impact *ST7* expression in these transformed cell lines. Real-time RT-PCR analysis showed that *ST7* mRNA expression was reduced 2- to 12-fold in cancer cell lines ( $P < 10^{-4}$ ) (Figure 5A). However, there was no correlation between PRMT5 protein levels and the extent of *ST7* mRNA repression. Remarkably, when *ST7* protein levels were measured by Western blotting, all trans-



**Figure 5** *ST7* is silenced in transformed lymphoid cell lines. (A) *ST7* transcription is repressed in lymphoid cancer cell lines as determined by real-time RT-PCR. This experiment has been repeated three times in triplicates. (B) *ST7* protein expression was analyzed by Western blotting using 20  $\mu$ g of RIPA extracts from either normal B cells, or the indicated transformed lymphoid cell lines.  $\beta$ -ACTIN levels were detected to ensure equal loading. (C) Fixed normal or transformed B cells were incubated with either pre-immune or immune anti-*ST7* antibody. *ST7* protein was visualized using goat FITC-labeled anti-rabbit antibody, whereas nuclei were stained with DAPI. Pictures were taken at  $\times 100$  magnification. (D, E) ChIP assays were performed on crosslinked chromatin from normal or transformed B cells using either preimmune (PI) or the indicated immune antibodies, and the retained DNA was amplified by real-time PCR using *ST7*-specific primers and probe. Fold enrichment with each antibody was calculated relative to the PI sample. Each ChIP experiment was repeated twice in triplicates.

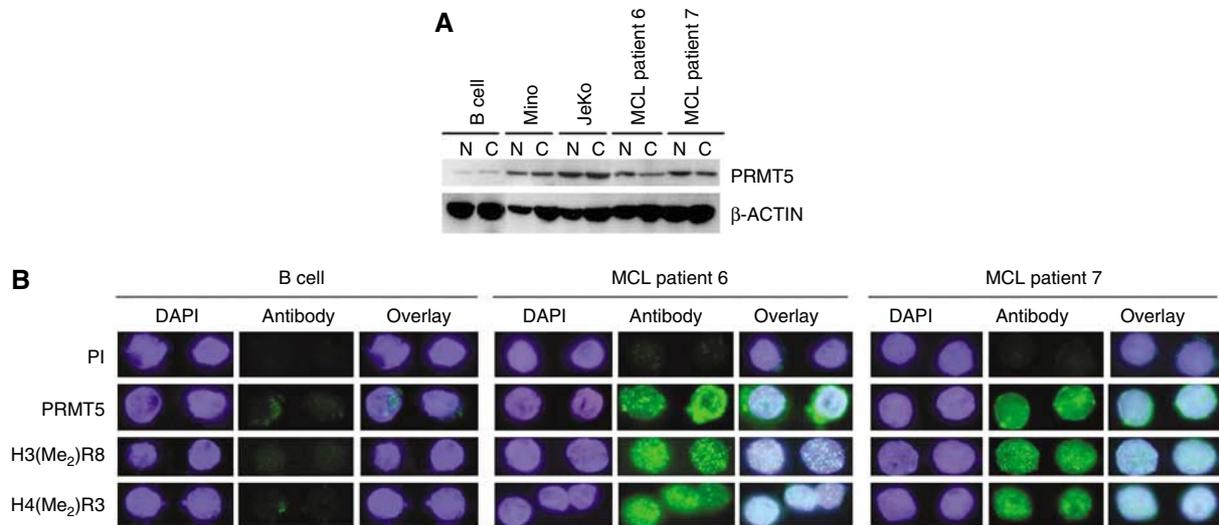
formed lymphoid cell lines lacked *ST7* expression, suggesting that there is an inverse relationship between PRMT5 and *ST7* protein levels (Figure 5B). To further confirm these results, we detected *ST7* protein by immunofluorescence in normal B cells as well as Mino and JeKo MCL cell lines. In accord with the Western blot data, *ST7* was detected in normal B cells, but not in transformed MCL cell lines (Figure 5C). It is worth

noting that expression of *ST7* was not detected when either nuclear or cytosolic extracts were used (data not shown); however, *ST7* was readily detectable when whole-cell lysates were prepared using RIPA buffer. These results and the immunofluorescence staining pattern in normal B lymphocytes suggest that *ST7* protein is insoluble and probably membrane-bound.

Expression of *ST7* mRNA was inhibited in all transformed lymphoid cell lines examined, suggesting that *ST7* promoter activity is altered in these cancer cell lines. To verify if PRMT5 is directly involved in inducing *ST7* repression, we conducted chromatin immunoprecipitation (ChIP) experiments using anti-PRMT5-specific antibodies (Figure 5D). ChIP analysis showed that PRMT5 recruitment to the *ST7* promoter was enriched 3- and 5.5-fold in transformed Mino ( $P = 10^{-3}$ ) and JeKo ( $P = 0.006$ ) MCL cell lines, respectively. Consistent with our previous findings in NIH3T3 cells, BRG1 recruitment to the *ST7* promoter was enhanced 1.6- and 3-fold in Mino ( $P = 10^{-2}$ ) and JeKo ( $P = 0.0007$ ), respectively, whereas BRM recruitment was unaffected. These results show that the BRG1-associated PRMT5 is involved in *ST7* transcriptional repression in transformed B cells. As PRMT5 can symmetrically methylate H3R8 and H4R3, we evaluated the methylation status of these residues at the *ST7* promoter (Figure 5E). We found that methylation of H3R8 is augmented 1.7- ( $P = 0.013$ ) and 2.7-fold ( $P < 10^{-4}$ ) in Mino and JeKo cells, respectively. Similarly, methylation of H4R3 is enriched 3.7- ( $P = 0.002$ ) and 2.5-fold ( $P < 10^{-4}$ ) in these MCL cell lines. We have also analyzed BRG1, BRM, and PRMT5 recruitment as well as H3R8 and H4R3 methylation at the *ST7* promoter in two other lymphoma cell lines, Daudi and Raji, and found similar results (Supplementary Figure 3). These findings show that increased expression of PRMT5 is directly involved in *ST7* gene repression, and that PRMT5 recruitment as well as H3R8 and H4R3 methylation are altered in transformed lymphoid cell lines.

### Expression of PRMT5 and its target gene *ST7* is altered in MCL clinical samples

We have found that PRMT5 protein levels are aberrantly increased in a wide variety of transformed lymphoid cells, including Mino and JeKo MCL cell lines, and as a consequence *ST7* expression is inhibited. To confirm our results and to assess whether the inverse relationship between PRMT5 and *ST7* exists in tumors isolated from MCL patients, we first measured *PRMT5* mRNA levels in several MCL clinical samples (Supplementary Figure 4). Our results demonstrate that MCL clinical samples exhibit reduced levels of *PRMT5* mRNA. Furthermore, when we analyzed PRMT5 protein expression in MCL clinical samples 6 and 7, which were not limiting, we found that its levels were elevated in the nuclear and cytosolic fractions (Figure 6A). More importantly, immunofluorescence analysis revealed that PRMT5 is overexpressed and that global symmetric methylation of H3R8 and H4R3 is proportionally enhanced in MCL patient samples 6 and 7 (Figure 6B). As PRMT5 can directly inhibit *ST7* transcription, we analyzed *ST7* mRNA and protein expression in more MCL patient samples. Real-time RT-PCR showed that *ST7* mRNA levels were reduced 2- to 30-fold ( $P < 10^{-4}$ ), and Western blot analysis indicated that *ST7* protein expression was severely inhibited in all MCL clinical samples examined (Figures 7A and B). In addition, immuno-



**Figure 6** MCL patients overexpress PRMT5 protein. (A) Nuclear and cytosolic extracts from normal CD19<sup>+</sup> B cells or MCL clinical samples 6 and 7 (20 μg) were analyzed by Western blotting using either anti-PRMT5 or control β-ACTIN antibody. (B) Immunofluorescence of normal B and transformed MCL cells from clinical samples 6 and 7 after staining with DAPI, PI, or antibodies to PRMT5, H3(Me<sub>2</sub>)R8, and H4(Me<sub>2</sub>)R3.

fluorescence staining of ST7 protein in MCL samples from patients 6 and 7 confirmed the Western blot results, and showed that ST7 protein expression is inhibited in MCL clinical samples (Figure 7C). Next, we analyzed BRG1, BRM, and PRMT5 recruitment to the *ST7* promoter in MCL patient samples 6 and 7 (Figure 7D). BRG1 recruitment was enriched 2- ( $P=0.002$ ) to 3.6-fold ( $P=0.006$ ), whereas PRMT5 recruitment was enhanced 8- ( $P=0.0002$ ) to 14-fold ( $P=0.003$ ) in MCL clinical samples 6 and 7. As a result of increased recruitment of BRG1-associated PRMT5 to the *ST7* promoter, symmetric methylation of H3R8 was augmented 3- ( $P=0.003$ ) to 7-fold ( $P=0.016$ ), and H4R3 methylation was elevated 4.6- ( $P=0.001$ ) to 5-fold ( $P=0.008$ ) in MCL clinical samples 6 and 7 (Figure 7E). Collectively, these results demonstrate that altered expression of PRMT5 directly affects H3R8 and H4R3 global methylation and triggers changes in target gene expression which may in turn contribute to MCL.

#### Knocking down PRMT5 alters the growth characteristics of transformed lymphoid cell lines

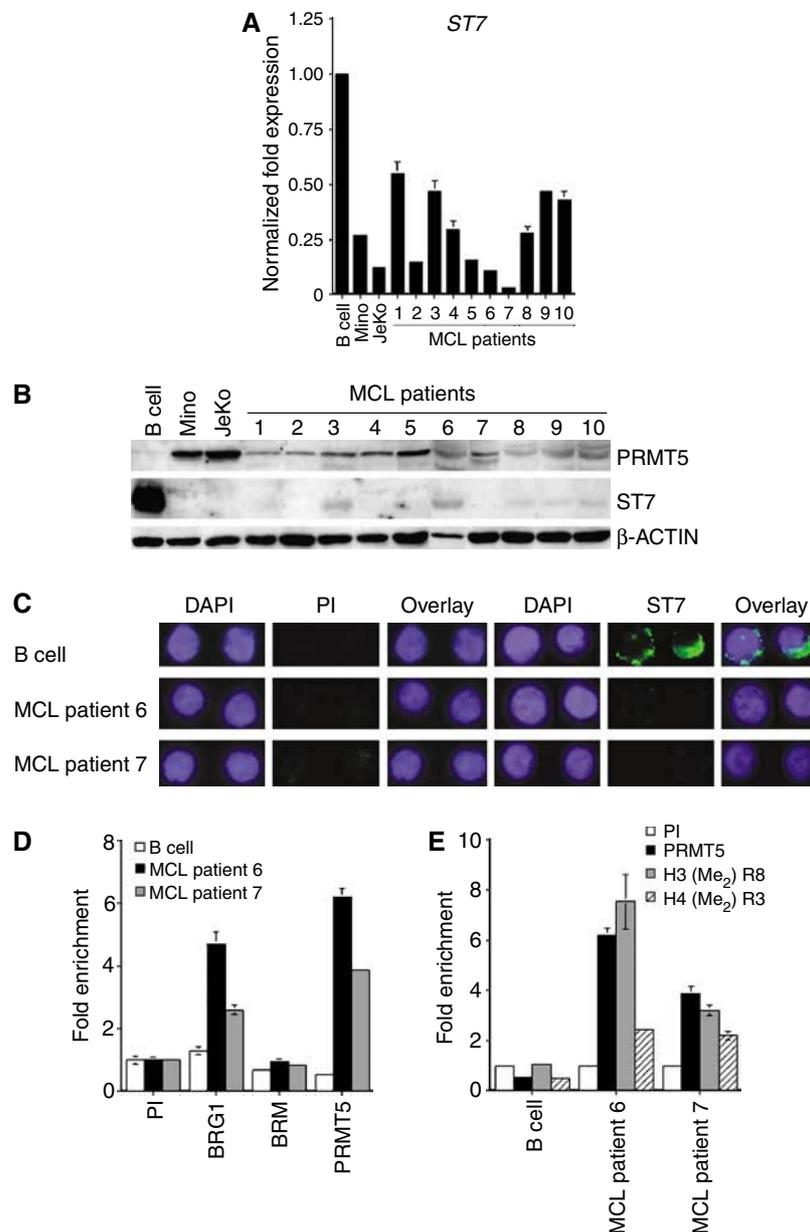
Previous work showed that overexpression of PRMT5 in NIH3T3 cells induces hyperproliferation, whereas knock-down of PRMT5 reduces cell growth and proliferation (Pal *et al*, 2004). As PRMT5 levels are high in MCL cell lines and clinical samples, we investigated whether reducing PRMT5 levels could alter MCL cell growth. To lower PRMT5 expression, JeKo cells were infected with either recombinant control or antisense (AS)-PRMT5 lentivirus, and PRMT5 protein expression was evaluated at different time points after infection (Figure 8A). PRMT5 protein levels were reduced significantly by day 4, whereas β-ACTIN expression was unaltered. When we monitored proliferation of control and AS-PRMT5 expressing JeKo cells, there was a noticeable 1.8-fold decrease in growth by day 6 ( $P < 10^{-4}$ ) (Figure 8B). To evaluate whether this decrease in cell growth and proliferation was a direct result of cell death and/or slow cell cycle progression, we measured both DNA content and BrdU incorporation of control and AS-PRMT5-infected cells. Our results indicated

that AS-PRMT5 cells incorporated BrdU 25% less efficiently than parental cells infected with control vector, and did not display any change in their cell cycle profile (data not shown). These results suggest that reducing expression of PRMT5 interferes with proliferation of transformed B cells.

We have shown that PRMT5 can directly regulate *ST7* transcription. Therefore, we measured *ST7* mRNA levels by real-time RT-PCR in control and AS-PRMT5-expressing JeKo cells (Figure 8C). Our results show that as PRMT5 protein levels decrease by day 6, transcription of *ST7* is induced 1.7-fold ( $P=10^{-3}$ ). Despite this transcriptional derepression, ST7 protein levels did not increase, suggesting that there might be other mechanisms involved in regulating ST7 protein expression (data not shown). Similar effects on cell proliferation and *ST7* mRNA expression were observed when PRMT5 was knocked down in Raji cells (Supplementary Figure 5). These findings provide more evidence that knocking down PRMT5 expression in cancer B cells impacts their growth characteristics, and that *ST7* expression is regulated post-transcriptionally.

## Discussion

Epigenetic modification of chromatin is the focus of intense studies, because there is a need to resolve many unanswered questions in cancer. To understand how post-translational changes of histones impact gene expression, and how perturbation of this process contributes to disease will certainly provide new avenues for cancer therapy. Our previous work showed that overexpression of PRMT5 in immortalized, but not transformed fibroblasts, induces their hyperproliferation and anchorage-independent growth, strongly suggesting that PRMT5 plays an important role in tumorigenesis (Pal *et al*, 2004). In this study, we showed that PRMT5 protein expression is induced in a wide variety of human lymphoid cancer cell lines, including patient-derived MCL cell lines as well as MCL clinical samples. We also linked misregulated expression of PRMT5 to aberrant expression of miR-92b and miR-96, and showed that the consequence of these changes



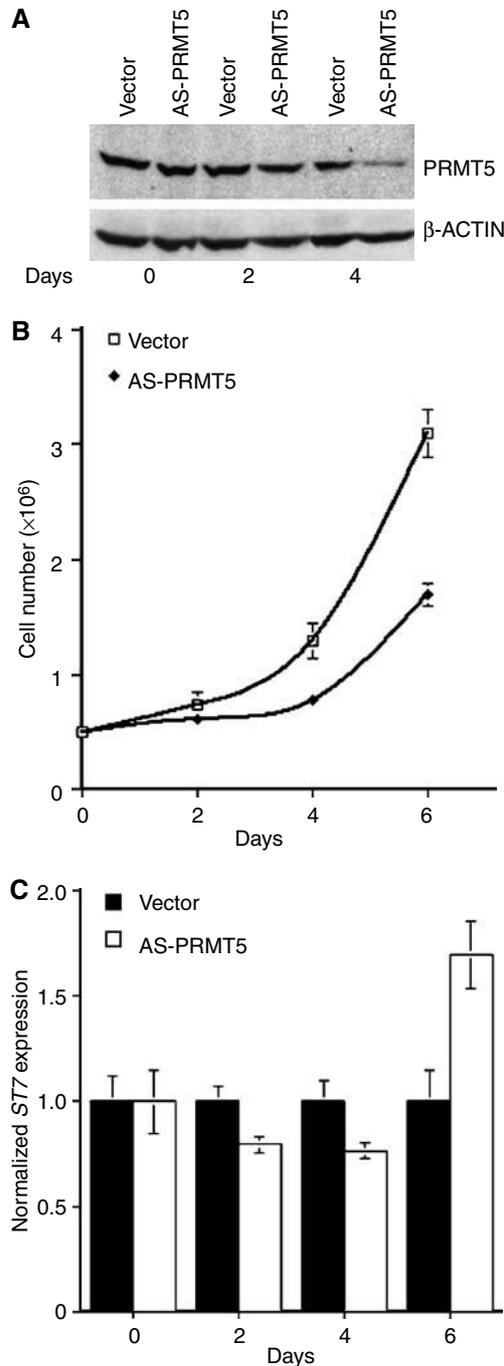
**Figure 7** Overexpression of PRMT5 correlates with *ST7* silencing in MCL clinical samples. (A) Real-time RT-PCR analysis of *ST7* mRNA expression in MCL patient samples 1–10. Expression of *ST7* was normalized using *GAPDH* as an internal control. (B) Western blot analysis was performed on 20  $\mu$ g of RIPA extracts from normal B cells, Mino, JeKo, and MCL clinical samples 1–10 using the indicated antibodies. (C) Immunofluorescence of normal B cells and MCL clinical samples 6 and 7 cells after staining with DAPI, PI, or immune anti-*ST7* antibody. Pictures were taken at  $\times 100$  magnification. (D, E) ChIP was performed on crosslinked chromatin from normal B cells and MCL clinical samples 6 and 7 using either PI or the indicated immune antibodies. Immunoprecipitated DNA was amplified by real-time PCR, and the fold enrichment with each antibody was calculated relative to the PI sample.

is increased global symmetric methylation of H3R8 and H4R3. More specifically, we demonstrated that expression of the PRMT5 target gene, *ST7*, is inhibited in human lymphoid cancer cell lines with altered PRMT5, miR-92b and miR-96 expression. Furthermore, our studies also show that knocking down PRMT5 expression reduces proliferation of transformed lymphoid cell lines, suggesting that reduced levels of PRMT5 are critical for normal cell growth.

**Aberrant expression of miR-92b and miR-96 is associated with enhanced PRMT5 translation in MCL**

Analysis of nuclear and cytosolic extracts from B cells and transformed lymphoid cell lines and MCL clinical samples

showed that PRMT5 protein expression is significantly higher in both compartments (Figures 1 and 6). We have also analyzed PRMT5 protein expression in various solid tumor cell lines, including glioma (U251, Gli3605), adenocarcinoma (HeLa S3, SW13), breast and lung carcinoma (BT549, A549), and hepatoma (HepG2) (Pal and Sif, unpublished), and we have found that global expression of PRMT5 protein is increased in both nucleus and cytosol in comparison to NIH3T3, Rat1a, and PC12 cell lines. Nuclear PRMT5 is associated with chromatin remodelers, including BRG1/BRM-based hSWI/SNF and NURD complexes, and is involved in transcriptional repression of cell cycle regulators and tumor suppressor genes (Pal *et al*, 2003, 2004; Guezennec



**Figure 8** Knocking down PRMT5 expression affects growth of transformed B cells. (A) Western blot analysis was performed on 20  $\mu$ g of RIPA extract from JeKo cells after infection with either vector or AS-PRMT5 lentivirus for 0, 2, and 4 days using anti-PRMT5 and control anti- $\beta$ -ACTIN antibodies. (B) Proliferation of JeKo cells infected with either control vector or AS-PRMT5 lentivirus. Cells were counted every two days for 6 days, and the experiment was repeated four times in duplicates. (C) *ST7* mRNA expression in lentivirus infected JeKo cells was evaluated by real-time RT-PCR at the indicated times. *ST7* mRNA expression is represented relative to control vector infected JeKo cells, and is normalized to *GAPDH*.

*et al*, 2006). Therefore, when PRMT5 levels are elevated either by overexpression or as it is the case in transformed cancer cells, it might repress transcription of key target genes and promote tumorigenesis.

Our findings show that increased expression of PRMT5 in transformed Mino and JeKo MCL cell lines is a direct result of enhanced *PRMT5* mRNA translation (Figures 2 and 3A). What is more striking is the finding that expression of two of the miRNAs predicted to bind to the *PRMT5* 3'UTR was reduced in patient-derived Mino and JeKo MCL cells as well as MCL clinical samples, and re-expression of miR-92b and miR-96 in two distinct lymphoma cell lines reduced PRMT5 translation *in vivo* (Figure 4A). Furthermore, mutation of the miR-92b or miR-96 seed sequence abolished PRMT5 translational inhibition *in vivo*, indicating that the observed repressive effects of miR-92b and miR-96 are specific. Our attempt to recapitulate translational inhibition of PRMT5 *in vitro* was not successful when wild-type miR-96 was used (data not shown). We have compensated for the lack of stability of *PRMT5* mRNA:miR hybrid molecules by increasing the complementarity beyond the seed sequence, and we have been able to specifically inhibit PRMT5 translation *in vitro* (Figure 4B). Further support for the involvement of miR-92b and miR-96 in regulating PRMT5 translation comes from our observation that the PRMT5 3'UTR can enhance luciferase translation in transformed JeKo cells with low miR-92b and miR-96 expression (Figure 4C). The fact that transfection of either wild-type miR-92b or miR-96 resulted in efficient decrease of PRMT5 expression in JeKo and Raji cells (Figure 4A), and the finding which showed that mutation of miR-92b- or miR-96-binding site improved luciferase translation (Figure 4C), argue that both miR-92b and miR-96 play an important role in regulating PRMT5 protein expression.

#### **Increased PRMT5 expression enhances global symmetric methylation of H3R8 and H4R3, and is accompanied by suppression of ST7**

Based on our previous work and current observations, association of PRMT5 with chromatin remodelers enhances its histone methyltransferase activity, and endows it with the ability to influence target gene expression so that the net outcome is to promote cell growth and transformation (Pal *et al*, 2004). As PRMT5 preferentially targets histones H3R8 and H4R3, we analyzed their methylation status in transformed MCL cells and show that these sites are highly methylated in patient-derived MCL cell lines and MCL clinical samples (Figures 1C and 6B). More specifically, when we analyzed histone methylation at the *ST7* promoter in MCL cells, we found that both H3R8 and H4R3 were hypermethylated (Figures 5 and 7). Whereas it is clear that recruitment of PRMT5 to the *ST7* promoter enhances H3R8 and H4R3 methylation, there appears to be no correlation between the levels of histone H3R8/H4R3 methylation and *ST7* transcriptional suppression (Figures 5 and 7).

It is well established that different post-translational modifications of histones can act either synergistically or antagonistically to specify transcriptional outcome (Fischle *et al*, 2003). For instance, histone H3K9 and H3K27 trimethylation marks colocalize at the repressed *Ubx* promoter (Ringrose *et al*, 2004). Therefore, it is possible that, in the case of *ST7* transcription, there are additional epigenetic marks involved in its regulation, which may vary between cell lines and clinical samples. A second possibility is that lack of correlation between H3R8/H4R3 methylation and *ST7* transcriptional suppression might arise due to differences in the expression of protein(s) that bind symmetrically methylated

H3R8 and H4R3. Recent studies have clearly shown that methylated histones can be recognized and bound by specific methyl-binding protein, and that this is important for transcriptional regulation. Good examples are provided by the WDR5 protein, which can bind to dimethylated H3K4 and support transcriptional activation of *HOXC8*, and by HP1, which can recognize trimethylated H3K9 and induce silencing (Hall *et al*, 2002; Wysocka *et al*, 2005). Thus, it is going to be important to determine whether additional silencing epigenetic marks are present at the *ST7* promoter, and whether there are specific factors that can bind symmetrically methylated H3R8 and H4R3.

We have determined that expression of *ST7* is regulated both at the transcriptional and translational levels. Knocking down PRMT5 expression in transformed JeKo and Raji cells induces *ST7* transcription, but does not affect *ST7* protein expression (Figure 8C and Supplementary Figures 2C and 5C). Similarly, transformed lymphoid cells that express low levels of *ST7* mRNA do not exhibit any detectable levels of *ST7* protein (Figures 5 and 7). We have determined that decreased expression of miR-92b and miR-96 leads to enhanced PRMT5 translation. Therefore, we cannot rule out the possibility that aberrant expression of *ST7*-specific miRNAs might be inhibiting *ST7* translation. More experiments are required to examine expression of *ST7*-specific miRNAs in normal and transformed B cells.

#### **Role of PRMT5 in mantle cell lymphomagenesis**

It is becoming more evident that misexpression and/or mutation of histone-modifying enzymes are associated with cancer etiology. For instance, the mixed lineage leukemia gene, *MLL1*, which methylates H3K4 and activates transcription, is frequently translocated in acute leukemias and as a consequence more than 50 different leukemogenic *MLL* fusion proteins are generated (Slany, 2005). Most of these *MLL* fusion proteins contribute to leukemogenesis by increasing *HOX* gene expression through mechanisms that involve histone hyperacetylation and H3K79 methylation (Slany, 2005). Another epigenetic mark that appears to be altered in various cancers involves the H3K27-specific methyltransferase, *EZH2*, which has been tightly linked to gene silencing. Overexpression of *EZH2* has been documented in prostate, breast, and gastric cancers and appears to correlate with the high degree of invasiveness of tumors (Varambally *et al*, 2002; Kleer *et al*, 2003; Matsukawa *et al*, 2006). In addition, increased expression of *EZH2* in Ramos lymphoma and prostate cancer cell lines induces their growth and hyperproliferation (Visser *et al*, 2001; Varambally *et al*, 2002). The role of histone arginine methylation in oncogenesis is not well understood.

Evidence in support of the role played by PRMT5 in cancer came from studies that showed that PRMT5 is frequently upregulated in gastric carcinoma (Kim *et al*, 2005). Furthermore, when MDA-MB-231 breast cancer cell line is transfected with ER- $\alpha$  and treated with 17 $\beta$ -estradiol, there is a decrease in cell proliferation, which is accompanied by reduced PRMT5 expression (Moggs *et al*, 2005). Our results show that PRMT5 undergoes translational upregulation in cancer cells of lymphoid origin, including MCL clinical samples. As a result, symmetric methylation of H3R8 and H4R3 is augmented. The impact of enhanced histone arginine methylation is misregulated gene expression, and in the case

of the PRMT5 target gene, *ST7*, there is a clear inhibition of transcription, which appears to be associated with cancer cell growth. We also show that knocking down PRMT5 expression in MCL and Burkitt's lymphoma cell lines reduces cell proliferation. Thus, it appears that PRMT5 is a key histone-modifying enzyme that controls cell growth by modulating expression of target genes through histone arginine methylation.

Elevated expression of PRMT5 can impact other pathways besides methylation of histones H3R8 and H4R3. Recently, PRMT5 was shown to interact with and methylate MBD2 that is associated with the NURD complex (Guezennec *et al*, 2006). The consequence of this methylation is reduced MBD2 binding to methylated DNA and inability to repress transcription (Tan and Nakielnny, 2006). Thus, it is possible that in addition to histone modification, PRMT5 promotes tumorigenesis by negatively modulating the activity of MBD2-based NURD complex. Therefore, it is going to be interesting to examine the methylation status of MBD2 in cancer cells that overexpress PRMT5, and to verify if expression of NURD target genes is altered in these cancer cells. Furthermore, our findings, which show that reducing expression of PRMT5 can inhibit cell proliferation, provide a good starting point to devise new strategies aimed at addressing the therapeutic relevance of targeting PRMT5.

## **Materials and methods**

#### **Plasmid constructions**

Plasmids were constructed as described in Supplementary data.

#### **Cell culture, B cell isolation, transfection, luciferase assay, lentivirus production, and cell infection**

Normal and transformed lymphoid cells were cultured in RPMI-1640 supplemented with 10–20% FBS. Normal B cells were isolated from tonsils, obtained from Children's Hospital through the Cooperative Human Tissue Network (CHTN) as described in Supplementary Data. Normal and transformed human B lymphocytes were collected under an IRB-approved and HIPAA-compliant protocol. Details for transfection, luciferase assay, lentivirus production, and cell infection are provided in Supplementary Data.

#### **Antibodies, Western blot, and immunofluorescence analyses**

For details see Supplementary data.

#### **Reverse transcription, real-time PCR, nuclear run on assay, polyribosome profiling, RNase protection assay (RPA), in vitro transcription, capping, polyadenylation, and translation**

Detailed protocols are described in Supplementary data.

#### **Chromatin immunoprecipitation assay**

ChIP experiments were performed using soluble crosslinked chromatin from approximately  $1 \times 10^7$  normal or transformed B cells as described previously, except that washing with the mixed micelle buffer was carried out twice (Pal *et al*, 2003, 2004). To assess recruitment to the *ST7* promoter, real-time PCR was performed on 3  $\mu$ l of eluted DNA in a 10  $\mu$ l reaction as described in Supplementary Data.

#### **Statistical analysis**

To statistically validate data generated using multiple samples within different groups, analysis of variance (ANOVA) was used to calculate the *P*-value. To identify differentially expressed genes or recruitment of various chromatin remodelers between two groups, paired *t*-tests were used to calculate the *P*-value. In all cases, GraphPad Prism4 software was used to generate *P*-values.

#### **Supplementary data**

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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