Tumorigenesis and Neoplastic Progression

Epigenetic Down-Regulation of the Tumor Suppressor Gene *PRDM1/Blimp-1* in Diffuse Large B Cell Lymphomas

A Potential Role of the MicroRNA Let-7

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PRDM1/Blimp-1, a master regulator for B cell terminal differentiation, is a putative tumor suppressor in diffuse large B cell lymphomas (DLBCL). Inactivating mutations of *PRDM1* have been previously identified in a subset of nongerminal center B cell–like (GCB) DLBCL. We investigated the presence of alternative mechanisms of down-regulating *PRDM1* in a cohort of 25 primary DLBCL and six DLBCL cell lines. While some DLBCL, predominantly the GCB-type, showed low levels of both *PRDM1α* mRNA and protein, presumably as a result of direct transcription repression, discordant expressions between the two were identified in a subset of DLBCL without *PRDM1* mutations, the primarily non-GCB type, consistent with translational down-regulation. This subset of DLBCL exhibits relatively high *PRDM1α* mRNA levels but low levels of PRDM1. Data obtained from expression analysis, luciferase reporter assays, and transfection experiments support a role of targeting of *PRDM1* by microRNA let-7 family in mediating this down-regulation. Let-7, in particular let-7b, is overexpressed in DLBCL relative to normal GCB cells, suggesting that it is deregulated. Thus, abnormal epigenetic down-regulation of *PRDM1* by let-7 and other microRNAs may represent an alternative mechanism of reducing normal PRDM1 function in a subset of DLBCL with relatively high *PRDM1α* mRNA expression and unmutated *PRDM1*. These findings provide further evidence for an important role of impairment of terminal B cell differentiation in DLBCL

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PRDM1/Blimp-1 is a master transcriptional regulator for terminal differentiation of B cells into plasma cells.[1–4] It also plays a critical role in effector and memory T-cell differentiation,[1,3,5] myeloid cell development,[6] dendritic cell development,[7] and epithelial cell differentiation.[8] Inactivating mutations that result in the generation of a severely truncated nonfunctional PRDM1 are found in about 20% of the diffuse large B cell lymphomas (DLBCLs) of the nongerminal center B cell (GCB) subtype[9,10] and also in about 19% of the primary DLBCL of the central nervous system.[11] These mutations are associated with deletion of the other paired *PRDM1* allele at chromosome 6q21. These findings are consistent with *PRDM1* being a DLBCL tumor suppressor gene and imply an important role of impairment of terminal B cell differentiation in DLBCL pathogenesis. However, many DLBCLs lack genetic alterations in *PRDM1*. Posttranscriptional down-regulation of *PRDM1* in DLBCL has been suggested, but no systematic evaluation was performed.[10] In this study, we investigated this possibility in detail by comparative and quantitative analysis of PRDM1 mRNA and protein expression in different subgroups of DLBCL. We concluded that low or absent PRDM1 expression is a common phe-

nomenon in DLBCL and documented the presence of translational down-regulation of PRDM1 in a subset of DLBCL by comparing *PRDM1* mRNA and protein levels in these tumors. Recently it was demonstrated that the low levels of PRDM1 in Reed-Sternberg/Hodgkin cells in Hodgkin lymphoma can be at least partially attributed to translation inhibition by microRNAs (miRNAs) inside the cells,[12] which are ~22 nt RNAs that regulate gene expression by inhibiting protein translation and/or promoting mRNA degradation through binding to specific target sites in the 3′ untranslated region (UTR).[13] In this study, we demonstrated that overexpression of let-7 may contribute to the down-regulation of *PRDM1* expression in DLBCL by translation inhibition.

Materials and Methods

Patient Tissue Samples, Cell Lines, and Normal B Cells

Frozen archival tissue of 25 *de novo* DLBCL cases were obtained according to the protocols approved by the Institutional Review Board of Weill Medical College of Cornell University. All cases were reviewed and classified according to World Health Organization classification. All cases show >80% of tumor content. Classification into GCB and non-GCB types was performed according to Hans et al[14] using immunohistochemical staining of paraffin-embedded formalin-fixed tissues sections against BCL6, CD10, and IRF4/MUM1. DLBCL cell lines (OCI-Ly1, OCI-LY7, SUDHL2, SUDHL6, and U2932) and myeloma cell line U266 were cultured in RPMI medium 1640 with 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA). OCI-Ly3 was grown in Iscove modified Dulbecco medium supplemented with 10% human serum (NABI Biopharmaceuticals, Boca Raton, FL). Normal B cell subsets were sorted from fresh tonsillar CD19+ B cells by flow cytometry into naïve B cells (IgD+CD38−), GBC (IgD−CD38+), and memory B cells (IgD−CD38−). Three independent isolates were obtained from each B cell subset. Plasma cells were purchased from Stem Cell Technologies (Vancouver, Canada). Myeloma cells were isolated from frozen bone marrow aspirate samples of five different cases by CD138 immunomagnetic positive selection (Stem Cell Technologies, Vancouver, Canada).

Nucleic Acid Extraction

Total RNA was extracted from frozen tissue sections or tissue culture cells using the mirVana miRNA Isolation Kit (ABI/Ambion, Texas, USA) following the manufacturer's protocol. High-molecular-weight genomic RNA was isolated using the salting-out method as described.

Quantitative Real-Time Reverse-Transcriptase PCR

Quantitative detection of *PRDM1α* mRNA was performed as previously described.[12] Conditions of qRT-PCR for measurement of CD3ε expression are identical to those used for *PRDM1*, except that the primers and probes for real-time detection were purchased from Applied Biosystems as predeveloped assays.

MicroRNA Cloning and Quantitation

MicroRNA cloning was performed as previously described.[15,16] Let-7a, let-7b, and let-7f expression were measured by Taqman MicroRNA Assay (Applied Biosystems) following manufacturer's recommended protocols, using U6 RNA for normalization as determined by modified Invader assays.[17] For each sample, three independent measurements were made with triplicates for each measurement. Relative levels were calculated with respect to let-7 content in normal plasma cells.

Antibodies

Monoclonal PRDM1 antibody clone ROS (ref) was kindly provided by Dr. Jose Francisco Garcia at Dako Diagnostics S.A., Barcelona, Spain. Other antibodies used were: PRDM1 (clone 3H2EB, Santa Cruz Biotechnology, Santa Cruz, CA), BCL-6 (clone PG-B6P, DAKO, USA, dilution 1:10), CD10 (clone 56C6, Vector Laboratories, Burlingame, CA, dilution 1: 30), MUM-1/IRF4 (polyclonal antibody, DAKO, USA, dilution 1:100).

Western Blotting and Immunohistochemistry

Immunoblotting was performed as previously described[12] on total or nuclear extracts using clone ROS (1:1) and clone 3H2EB (1:200), respectively. Quantification of PRDM1α expression in the Western blot was done by densitometry and normalized with β-actin or lamin B loading control.

Immunohistochemistry for PRDM1 on paraffin-embedded formalin-fixed tissues was performed using the PRDM1 monoclonal antibody (clone ROS, 1:8) as described,[18] except that primary and postprimary incubations were performed using the Bond Polymer Refine Detection System (Vision Biosystems, Bannockburn, IL).

Luciferase Reporter Assays

293T cells were transfected with 20 nmol/L of let-7a, let-7b, let-7f, or miRNA negative control (purchased from Ambion, Austin, TX) in 24-well plates using Lipofectin (Invitrogen). These mmiRNA analogs were cotransfected with 25 ng of pSIC.PRDM1.3′ UTR.538–2419 (wild-type or mutant). Construction of these plasmids has been described in detail.[12] Briefly, the wild-type plasmid carries nucleotides 538 to 2419 of the *PRDM1* 3′ UTR downstream of the reporter gene. The mutant plasmid carries the same 3′ UTR genomic fragment, except that the fifth and sixth positions (from the 3′ end) of the putative let-7 binding site are mutated. Luciferase activities were measured 24 hours after transfection as described.

A

B
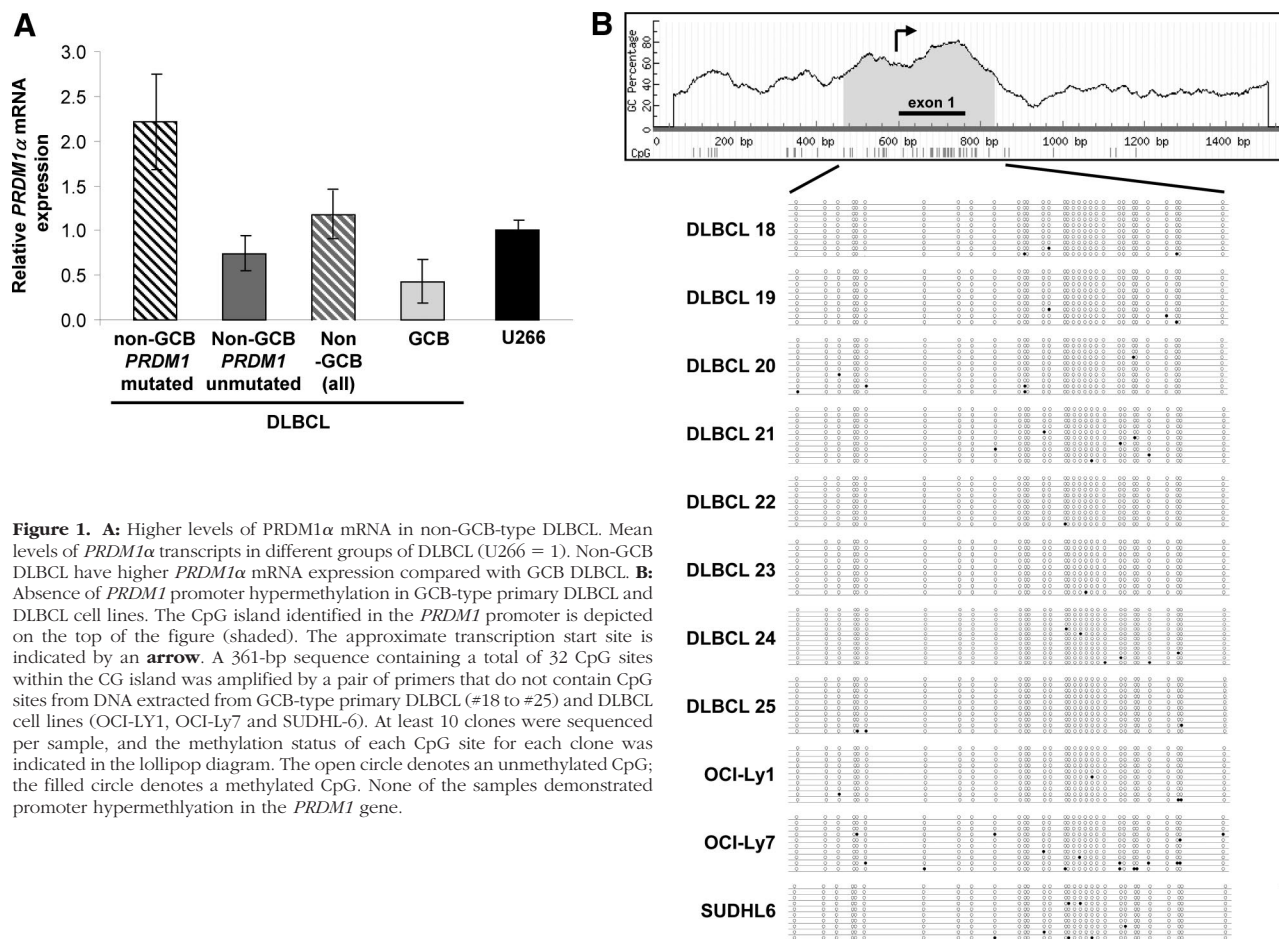
Figure 1. A: Higher levels of PRDM1α mRNA in non-GCB-type DLBCL. Mean levels of *PRDM1α* transcripts in different groups of DLBCL (U266 = 1). Non-GCB DLBCL have higher *PRDM1α* mRNA expression compared with GCB DLBCL. **B:** Absence of *PRDM1* promoter hypermethylation in GCB-type primary DLBCL and DLBCL cell lines. The CpG island identified in the *PRDM1* promoter is depicted on the top of the figure (shaded). The approximate transcription start site is indicated by an **arrow**. A 361-bp sequence containing a total of 32 CpG sites within the CG island was amplified by a pair of primers that do not contain CpG sites from DNA extracted from GCB-type primary DLBCL (#18 to #25) and DLBCL cell lines (OCI-LY1, OCI-Ly7 and SUDHL-6). At least 10 clones were sequenced per sample, and the methylation status of each CpG site for each clone was indicated in the lollipop diagram. The open circle denotes an unmethylated CpG; the filled circle denotes a methylated CpG. None of the samples demonstrated promoter hypermethlyation in the *PRDM1* gene.

Bisulfite Sequencing

Promoter methylation in *PRDM1* was assessed by bisulfite sequencing. Bisulfite-modified DNA from GCB-type primary DLBCL and DLBCL cell lines was amplified using a primer pair that contains no CpG sites. These primers were designed using the MethPrimer program[19] and have the following sequences: 5′-GTGTTAGTAATTT-GGGGGAAAGTTT-3′, forward primer; 5′-CAAACAAA-TATCCAACATCTAAAAAAAA-3′, reverse primer. This amplicon derived from these primers spans a sequence of 361 bp that contains 32 CpG sites within a CG island predicted at the *PRDM1* promoter. The amplified sequence was cloned into pGEM-T cloning vector (Promega, Madison, WI), and at least 10 clones were sequenced per sample. The methylation status of each CpG site for each clone is indicated.

Results

Discordance of PRDM1 *mRNA and Protein Expressions in Primary DLBCL*

We analyzed *PRDM1α* mRNA expression by quantitative real-time PCR in five DLBCL with *PRDM1* inactivating mutations, 12 non–GCB-type DLBCL without *PRDM1* mutations, and eight GCB-type DLBCL (see Supplemental

Table S1 at *http://ajp.amjpathol.org*). Overall, the non-GCB DLBCL cases tend to have a higher *PRDM1α* mRNA compared with GCB DLBCL ($P = 0.08$). In addition, the *PRDM1*-mutated subset of the non-GCB DLBCL cases have significantly higher levels of *PRDM1α* mRNA compared with the other two groups ($P = 0.005$) (Figure 1A). To ensure that the observed differences in *PRDM1a* mRNA are intrinsic to B cells and not due to variation in the quantity of reactive T cells in the biopsy specimen, the T cell contents of these representative cases were estimated by quantitative RT-PCR for CD3, which showed that they were low and similar among cases (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). The low level of PRDM1α expression in GCB-type DLBCL is not a result of promoter hypermethylation (Figure 1B).

PRDM1α protein expression in these DLBCL cases was assessed by immunohistochemistry using the monoclonal anti-PRDM1 antibody. Though this antibody recognizes both PRDM1 isoforms, this immunohistochemical analysis was essentially a measure of the quantity of the PRDM1α isoform because PRDM1β is a minor isoform in DLBCL.[20] Under our immunohistochemical conditions, all but one DLBCL showed PRDM1 positivity in less than 20% of tumor cells, with staining intensities often distinctly weaker compared with the myeloma U266 cells as well as the reactive T cells (see examples in Figure 2A). A previous study also demonstrated negative or weak

A

B
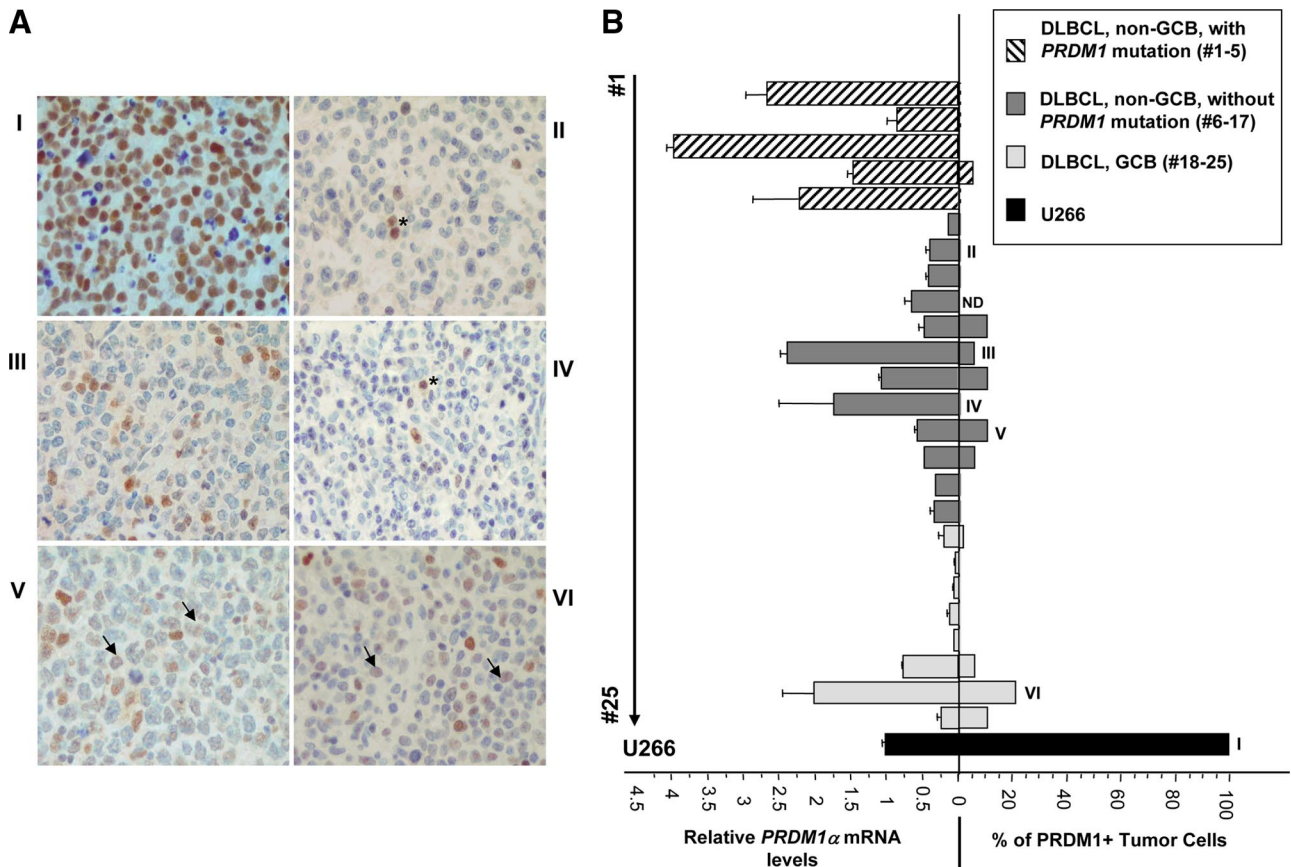
Figure 2. Discordance between *PRDM1* mRNA and protein expressions in DLBCL. **A:** Examples of immunohistochemistry for PRDM1 in U266 myeloma cells (I) and selected DLBCL cases (II–VI). Under these immunohistochemical conditions, most of the DLBCL cells are negative for PRDM1. In cases II and IV, the tumor cells are essentially negative for PRDM1. Scattered small PRDM1(+) T cells serve as internal controls (**asterisks**). In case III, a small number of PRDM1(+) tumor cells are identified. Cases IV and V show higher percentage of PRDM1(+) lymphoma cells, but most of them are only weakly positive (**arrows**). In contrast, U266 cells show uniform strong PRDM1 staining. **B:** *PRDM1α* mRNA and the corresponding protein expression in 25 DLBCL cases. The *PRDM1α* mRNA levels were expressed relative to the myeloma cell line U266 (arbitrarily set as 1), which highly expressed the *PRDM1* protein. The *PRDM1* protein levels were determined by immunohistochemistry on formalin-fixed paraffin-embedded sections and expressed as percentage positivity of tumor cells. Cases that were illustrated in **A** were marked. ND, not determined.

PRDM1 staining in most DLBCL.[18] Comparison of these immunohistochemistry results with the RNA expression data revealed a discordance between *PRDM1α* mRNA and protein expressions in virtually all of the non–GCB-type DLBCL. The lack of detectable full length PRDM1α expression in those DLBCL with *PRDM*1 mutations, which have high levels of *PRDM1α* mRNA, is expected as these mutations are inactivating and generate severe protein truncations.[9,10] In the other cases without *PRDM1* mutations, the degree of *PRDM1α* mRNA expression does not translate to the same extent of *PRDM1* protein expression (Figure 2B), suggesting a translational or posttranslational down-regulation. This is well illustrated by cases #7, 11, 13, and 14 (II to IV, Figure 2B), in which the levels of *PRDM1α* mRNA is at least 40% that of U266 but the PRDM1 levels are markedly lower relative to U266. Overall, in the *PRDM1*-unmutated non-GCB DLBCL group, which has a mean *PRDM1α* mRNA level of 74% (±19%) that of U266, the mean percentage of PRDM1(+) tumor cells is only less than 5%.

Contrary to the non-GCB DLBCL, at least half of the GCB DLBCL (for example cases #19 to 22) demonstrated synchrony between levels of *PRDM1α* mRNA and *PRDM1*

protein. The mean PRDM1α mRNA expression among these four cases is about 7% that of U266, and the *PRDM1* protein expression is barely detectable. These results imply that transcription repression is the major mode of *PRDM1* regulation in many of the GCB DLBCL. However, in some of the GCB DLBCL, for example, case #24 (VI, Figure 2B), discordance between *PRDM1* mRNA and protein expression is also observed.

Overexpression of Let-7 in Primary DLBCL

MiRNAs are small RNA regulators that are capable of modulating gene expression posttranscriptionally. Their functions depend on perfectly complementary interactions between the seed sequence at the 5′ end of the miRNAs and the 3′ region of the target sites.[13] We examined the possibility that the miRNA milieu in DLBCL contributes to the posttranscriptional down-regulation of *PRDM1* expression by searching for miRNAs that has the potential to target *PRDM1* in DLBCL, as evidenced by a high expression and the presence of predicted miRNA binding sites in the *PRDM1* 3′ UTR. The miRNA expres-

A

B

C
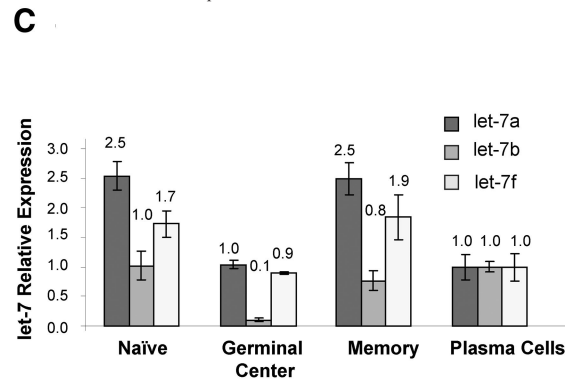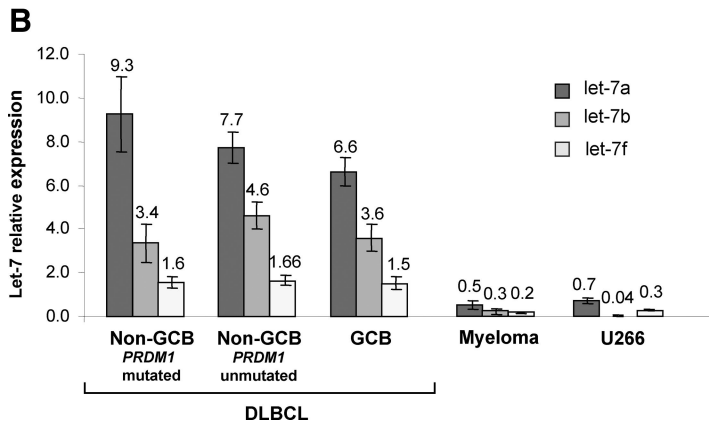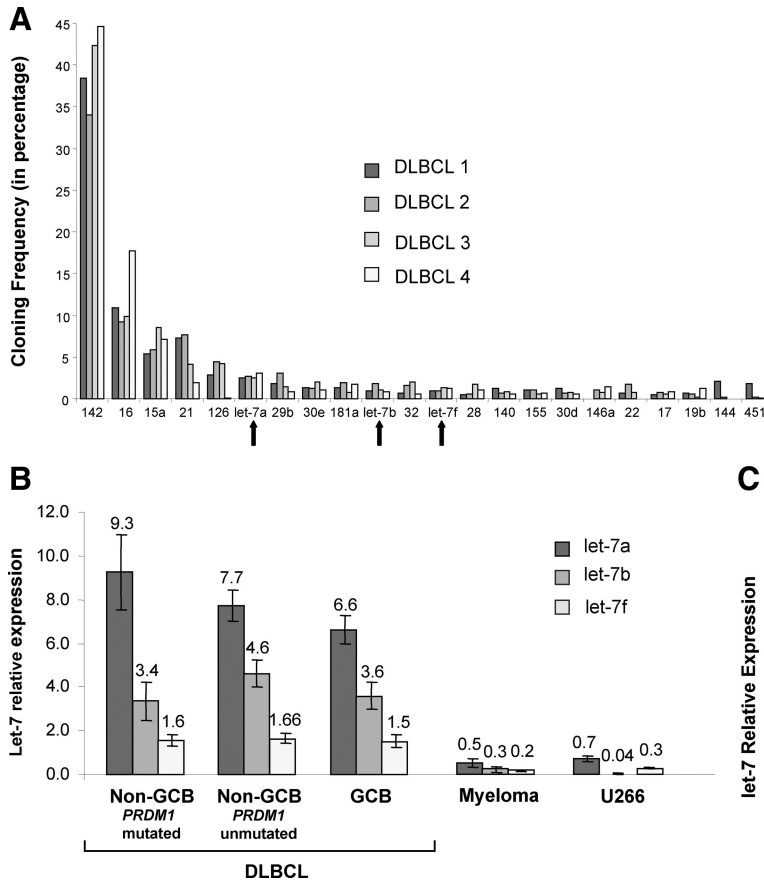
Figure 3. Let-7 is overexpressed in DLBCL. **A:** MicroRNA profiles of four DLBCL samples based on direct miRNA cloning. The cloning frequency of each miRNA is shown as a percentage of total miRNA clones isolated for each DLBCL sample. MiRNA that represent >0.5% of the total miRNA population on average was indicated. Let-7a, let-7b, and let-7f (marked by **arrows**) were among the most highly expressed miRNA in DLBCL. **B:** Let-7a, let-7b, and let-7f expressions in DLBCL (25 cases), plasma cell myeloma (5 cases), and U266 myeloma cell line (plasma cells = 1 for each let-7 species). Overall, DLBCLs demonstrated significantly higher expressions of let-7 compared with U266 and plasma cells myeloma. Let-7 expressions, however, were similar among the DLBCL subgroups. Mean values are indicated on top of each bar. **C:** Let-7a, let-7b, and let-7f expressions in normal B cell subsets. Germinal center B cells have significantly lower levels of let-7 ($P = 0.004, 0.03, 0.03$ for let-7a, let-7b, and let-7f, respectively) relative to naïve and memory B cells. DLBCLs (see **B**) showed overexpression of let-7 relative to germinal center B cells. Mean values are indicated on top of each bar.

sion profiles for four primary DLBCL cases have been established by direct miRNA cloning (Figure 3A).[15] We identified from this analysis let-7 as a potential miRNA candidate to mediate *PRDM1* down-regulation in DLBCL. Three members of the let-7 family, let-7a, let-7b, and let-7f, are among the most highly expressed miRNAs in these four DLBCL samples. An evolutionarily conserved let-7 binding site is present in the *PRDM1* 3′ UTR. Moreover, let-7a has been previously shown to down-regulate *PRDM1* in Hodgkin lymphoma cell lines by translational inhibition.[12]

We confirmed the high levels of let-7 expression in DLBCL by measuring let-7a, let-7b, and let-7f levels in the current series of DLBCLs, as well as U266, plasma cell myeloma, and normal B cell subsets for comparison, using quantitative real-time PCR. In line with the miRNA profiling results, let-7a, let-7b, and let-7f are highly expressed in DLBCL, with levels 11, 115, and fivefold that of U266 ($P = 0.001, 0.007$ and 0.01) and 14, 16, and eightfold that of plasma cell myeloma ($P < 0.0001, = 0.02, <0.0001$, respectively; Figure 3B). There is no significant difference in let-7 expressions among the three DLBCL subgroups.

Let-7 expressions vary in B cells at different stages of differentiation (Figure 3C). The expression levels of let-7 are similar between naïve and memory B cells. All three members of let-7 are down-regulated in GC B cells relative to naïve B cells, with more marked decrease (~10-fold) in let-7b ($P = 0.004$ for let-7a; $P = 0.03$ for let-7b and

let-7f). There is a significant increase in let-7b ($P = 0.001$) as GC B cells differentiate into plasma cells, while differentiation from GC B cells into memory cells is associated with increased expression in all three let-7 members ($P = 0.007$ for let-7a; $P = 0.02$ for let-7b; $P = 0.09$ for let-7b). Comparison of let-7 expression between DLBCLs and normal GC B cells showed significant overexpression in the former. Overall, the levels of let-7a, let-7b, and let-7f in DLBCL are 7.7-, 41-, and 1.8-fold that of normal GC B cells, respectively ($P = 0.0003, 0.0015$, and 0.09).

Expression of PRDM1 and Let-7 in DLBCL Cell Lines

PRDM1 mRNA and protein expressions were also examined in DLBCL cell lines (Figure 4, A and B). In line with the observations for primary DLBCL, PRDM1α mRNA was expressed at barely detectable levels in GCB-type DLBCL cell lines (OCI-Ly1, OCI-Ly7, and SUDHL-6) but at much higher levels in DLBCL cell lines of the activated B cell (ABC) type (OCI-Ly3, SUDHL-2 and U2932). The low levels of *PRDM1*α mRNA in the GCB-type DLBCL cell lines is not due to promoter hypermethylation (see Figure 1B). In concordance with their low levels of *PRDM1*α mRNA transcription, *PRDM1*α protein was undetectable in the GCB-type DLBCL cell lines. However, we noted discordance in *PRDM1*α mRNA and protein expressions in two of three ABC-type DLBCL cell lines (OCI-Ly3 and
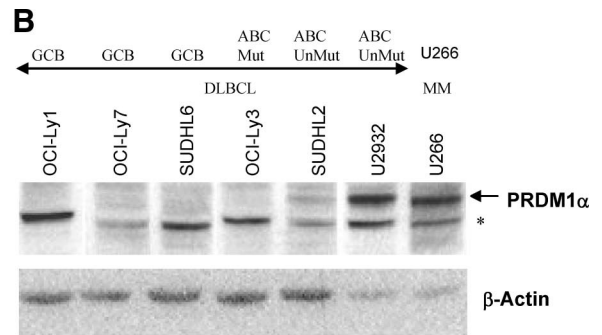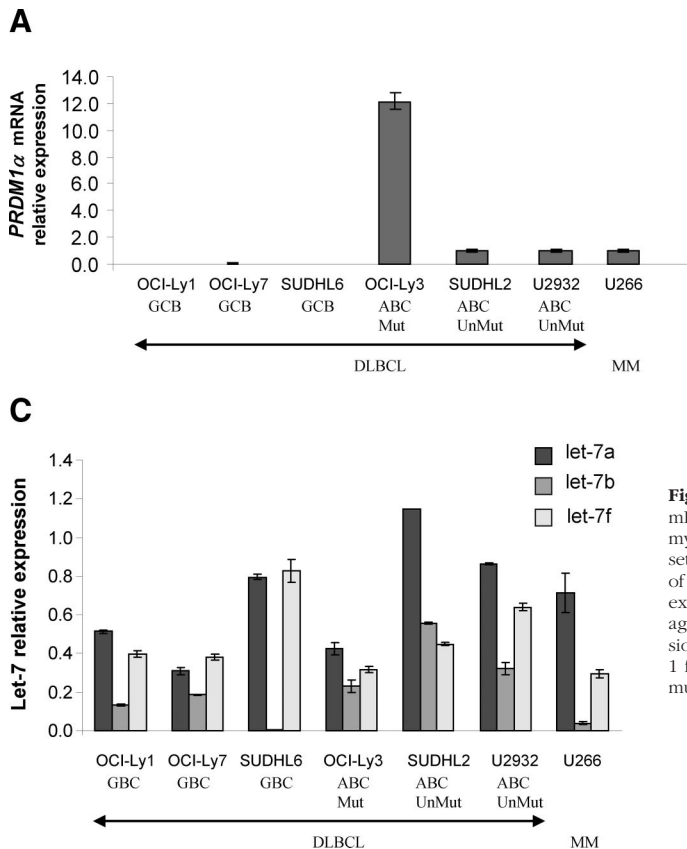
Figure 4. Expression of *PRDM1α* and let-7 in DLBCL cell lines. **A:** PRDM1α mRNA expression by real-time PCR analysis in six DLBCL cell lines and the myeloma cell line U266. The levels were expressed relative to U266 (arbitrarily set as 1). The *PRDM1α* transcript detected in OCI-Ly3 was truncated because of a rearrangement in the *PRDM1* gene. **B:** Western blot analysis of total cell extracts prepared from the above cell lines using the monoclonal antibodies against PRDM1. The nonspecific band is indicated by an **asterisk**. **C:** Expression of let-7a, let-7b, and let-7f in DLBCL cell lines and U266 (plasma cells = 1 for each let-7 species). For **A**, **B**, and **C,** the DLBCL subtypes and PRDM1 mutation status for the DLBCL cell lines are indicated.

SUDHL-2). The absence of PRDM1α in OCI-Ly3 can be attributed to the presence of inactivating mutations that resulted in premature translation termination and production of severely truncated nonfunctional protein.[9,10] The existence of discordance in *PRDM1* mRNA and protein expression in SUDHL-2 is evident when compared with U2932 and U266 cell lines. Though SUDHL-2 has a similar *PRDM1α* mRNA level as U2932 and U266, SUDHL2 has a much lower expression of *PRDM1α* protein compared with the other two cell lines. Sequencing of the *PRDM1* coding region revealed no mutations in SUDHL-2 or U2932. These results support the presence of translational or posttranslational down-regulation of *PRDM1* in the SUDHL-2 cell line and corroborate our findings in primary DLBCL cases.
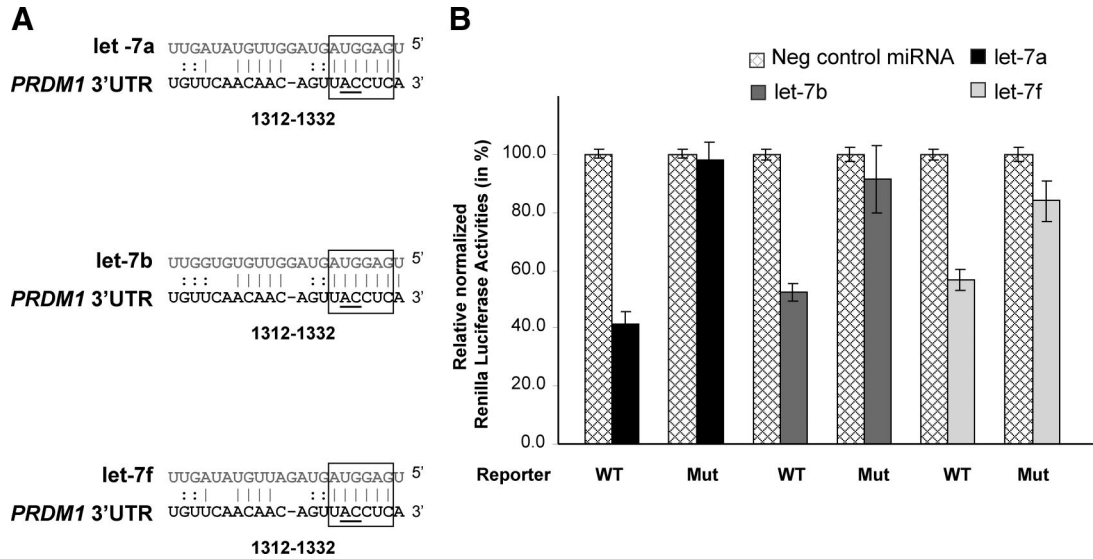
Examination of let-7 expression in DLBCL cell lines (Figure 4C) showed that let-7 is present at overall much lower levels in these cell lines compared with primary DLBCL cases (Figure 3B). Highest overall let-7 expressions are found in SUDHL-2, which shows discordance between *PRDM1* mRNA and protein expressions. Compared with normal GCB (Figure 2B), we observed a two- to sixfold overexpression of let-7b in four of the six DLBCL cell lines. Let-7b expression in SUDHL-2 is the highest among all of the cell lines and is almost twice that of U2932 ($P = 0.02$), which has similar levels of *PRDM1α* mRNA as SUDHL-2 but lower levels of *PRDM1* protein. Let-7a level in SUDHL-2 is also higher compared with U2932. These findings support a contribution of let-7, in

particular let-7b, in down-regulating *PRDM1* by translation inhibition in DLBCL cell lines.

Targeting of PRDM1 by Let-7

Let-7a has been shown to repress luciferase reporter activities by translation repression through specific binding to its predicted evolutionarily conserved target site in *PRDM1* 3′ UTR.[12] Let-7b and let-7f share the same target site as let-7a, with identical seed pairing but slightly different pairing at the 3′ portion of the miRNAs (Figure 5A). To confirm that let-7b and let-7f can also functionally target *PRDM1* 3′ UTR, luciferase assays were performed in 293T cells using *PRDM1* 3′ UTR with wild-type or mutated let-7 binding site. Transfection of let-7a, let-7b or let-7f all repress luciferase activities by about 50 to 60%. This repression can be relieved by mutating the seed sequence of the putative let-7 binding site (Figure 5B). Thus, these results indicate that let-7a, let-7b and let-7f can all specifically interact with the *PRDM1* 3′UTR and down-regulate gene expression.

Previously, we have shown that transfection of let-7a into U266 myeloma cells can repress endogenous *PRDM1* protein expression.[12] To determine whether transfection of let-7 can also regulate *PRDM1* in DLBCL cells, control miRNA, let-7a, or let-7b were transfected into U2932 cells and PRDM1α expression was measured by Western blotting 24 hours after transfection (Figure 6).

A

let -7a   UUGAUAUGUUGGAUGAUGGAGU 5'
          ::| ||||| ::||||||
PRDM1 3'UTR   UGUUCAACAAC–AGUUACCUCA 3'
          1312-1332

let-7b   UUGGUGUGUUGGAUGAUGGAGU 5'
          ::: ||||| ::||||||
PRDM1 3'UTR   UGUUCAACAAC–AGUUACCUCA 3'
          1312-1332

let-7f   UUGAUAUGUUAGAUGAUGGAGU 5'
          ::| |||| ::||||||
PRDM1 3'UTR   UGUUCAACAAC–AGUUACCUCA 3'
          1312-1332

B

Figure 5. Let-7 targets *PRDM1* 3′ UTR. **A:** Pairing between let-7 family (let-7a, let-7b, and let-7f) and their putative target site in *PRDM1* 3′ UTR. The highly conserved 6-bp "seed" pairing is highlighted by **open boxes**. The numbers indicate the locations of the putative binding sites in the *PRDM1* 3′ UTRs downstream from the *PRDM1* stop codon. The nucleotides of the let-7 target site that were mutated (from AC to GT) to disrupt the "seed" pairing are **underlined**. **B:** Reporter plasmids harboring *PRDM1* 3′UTR with wild-type sequence (WT) or point mutations in the let-7 target site (MUT) were cotransfected into 293T cells with let-7a, let-7b, or let-7f (20 nmol/L, Ambion), or with miRNA Negative Control oligonucleotides (20 nmol/L, Ambion). Luciferase activities (in triplicates) were measured 24 hours after transfection. *Renilla* luciferase activities were normalized against firefly luciferase activities, and mean normalized *Renilla* luciferase activities (±SE) from three independent experiments were determined and expressed relative to control values. Transfection of let-7 members resulted in about 40% to 60% of inhibition of reporter gene expression, which was relieved by mutations in let-7 binding site.

Both let-7a and let-7b repress endogenous PRDM1 expression in U2932 cells by about 40% relative to negative controls. Let-7f also has repressive effects, but to a lesser extent. These results indicate that let-7 can also target endogenous PRDM1 in DLBCL cells.
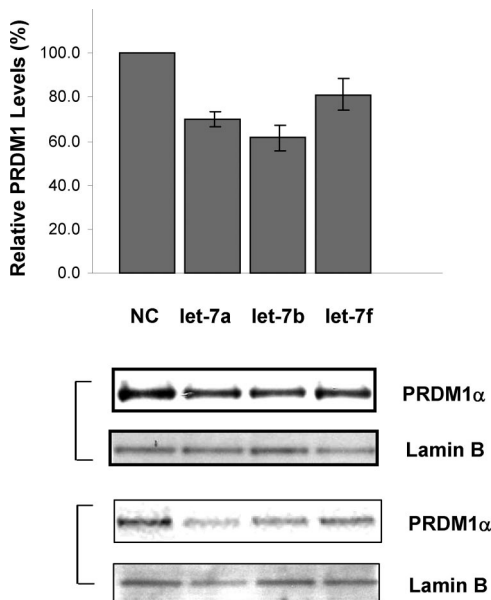
Figure 6. Let-7 down-regulated PRDM1 expression in U2932 DLBCL cell line. Control miRNA (NC), let-7a, and let-7b were transfected into U2932 cells by nucleofection. Nuclear extracts were prepared from each transfectant after 24 to 48 hours, and PRDM1α levels were measured by Western blot analysis using the anit-PRDM1 monoclonal antibody. Lamin B was used as a loading control. Normalized PRDM1α levels were expressed as a percentage relative to the negative control transfectants. The histogram was derived from two (let-7f) or four independent transfection experiments (let-7a and let-7b). Two representative Western blots are also shown.

Discussion

The expression of *PRDM1α* mRNA in DLBCL is heterogeneous, which is probably a result of the difference in signals and activities of transcription factors that regulate *PRDM1* expression.[1,21–25] Signals that can activate *PRDM1* expression include those from cytokines (IL5, IL6, IL-10, and IL-21), toll-like receptors, and antigen receptors. AP-1, NF-κB, IRF4/MUM1, STAT3, PU1, and C/EBPβ induce *PRDM1* transcription, while BCL6, PAX5, and BACH2 repress *PRDM1* transcription. In the absence of promoter hypermethylation, the low level of *PRDM1* mRNA present in some primary GCB-type DLBCL cases and GCB-type DLBCL cell lines is most likely due to transcription repression, for example, by BCL-6.[26] The higher *PRDM1α* mRNA induction in the primary non-GCB DLBCL cases and in the ABC-type DLBLC cell lines may be a result of higher NF-κB activities.[27]

Despite the heterogeneity in *PRDM1α* mRNA levels, we observed in general a relatively low *PRDM1α* protein expression in primary DLBCL cases. Previously, Garcia et al[18] has analyzed PRDM1 expression in a series of more than 200 DLBCL cases and observed negative or weak PRDM1 expression in most DLBCL. However, they also identified a group of DLBCL that demonstrated stronger PRDM1 expression. We were not able to detect these "high" PRDM1 expressors in our DLBCL series despite our ability to detect high PRDM1 expression in the normal GCB subset in reactive germinal centers and in the myeloma cell line U266 using identical immunohistochemical conditions throughout our study. The difference in results between the two studies may be due to case sampling and/or differences in immunohistochemical conditions. Because the study by Garcia et al did not

explicitly compare PRDM1 expression in these "high" expressors with reference standards such as multiple myeloma it would be difficult to assess accurately the actual PRDM1 content in those DLBCL. We did identify a DLBCL cell line (U2932) that has similarly high PRDM1 level as U266 based on Western blot analysis. Interestingly, U2932 is CD138(+),[28] indicative of plasmacytic differentiation. This finding suggests that those DLBCL with plasmacytic differentiation may tend to have higher levels of PRDM1 and provides additional supporting evidence for the established role of PRDM1 in plasma cell differentiation. In our series of primary DLBCL cases, none of the cases appear to demonstrate plasmacytic differentiation based on morphological evaluation and CD138 expression.

Comparative analysis of *PRDM1α* mRNA and protein in primary DLBCL cases and DLBCL cell lines demonstrated two distinct patterns. The first is present in a subset of GCB DLBCL, in which low *PRDM1α* mRNA expression is associated accordingly with low levels of *PRDM1* protein. There are exceptions though (for example, in case #24), which may be ascribed to the possibility of misclassification of these cases to GCB-type by immunohistochemistry. The second one is present in the vast majority of non-GCB DLBCL and also in some GCB-DLBCL, which demonstrated discordance between *PRDM1* mRNA and protein expressions. This discordance can be due to inactivating deletions and point mutations or translational/posttranslational down-regulation. The great majority of these DLBCL express IRF4/MUM1. Hence the low levels of PRDM1 seen in this group of DLBCL imply a pathogenetic dissociation between PRDM1 and IRF4 expressions, because these two transcription factors are normally co-expressed in GC centrocytes that are committed to plasma cell differentiation, and both factors are required for the generation of competent plasma cells.[29,30] Similar to primary DLBCL, discordance between *PRDM1* mRNA and protein is also evident in the DLBCL cell lines OCI-Ly3 and SUDHL-2. The former has mutations that inactivate PRDM1,[9,10] while the latter is likely due to translational/posttranslational regulation.

Our current and previous studies support *PRDM1* as a direct target of let-7 miRNAs. In line with our findings, *Blimp-1/prdm1* was demonstrated by elegant genetic experiments to be an *in vivo* target for let-7 in primordial germ-cell development in mice.[31] Down-regulation of *PRDM1* by let-7 appears to be mediated by translation inhibition.[12] Although let-7 has also been shown to be able to promote mRNA degradation,[32] this effect may be dependent on target genes and cell types. For example, let-7 inhibits translation of RAS with no effects on *Ras* mRNA levels,[33,34] while it primarily promotes mRNA degradation of HMGA2.[35] Its regulatory effects on c-*myc* vary depending on cell types. In colon cancer cells, it primarily inhibits translation of c-MYC,[34] while in Burkitt lymphoma cells, let-7 can down-regulate both c-*myc* mRNA and protein expressions.[36]

Let-7 members have been demonstrated by microarray analysis to exhibit temporal changes during B cell differentiation,[37,38] implying their role in this process.

Consistent with these results, our study using qRT-PCR also shows down-regulation of let-7 members in germinal center B cells. Let-7, in particular let-7b, appears over-expressed in DLBCL relative to normal GCB cells, implying that it may function as an oncogene in DLBCL. A tumor suppressor role of let-7 has been well documented in many human cancers by its ability to target several oncogenes, inhibit mitogenic pathways, and affect cell differentiation and apoptosis.[39] However, the tumor suppressor functions of let-7 described to date are limited to solid tumors and may not be applicable in lymphoid malignancies. There are also reports that suggest an oncogenic function for let-7.[40,41] Thus, it is conceivable that let-7 may have pleiotropic cell-type specific biological effects depending on the milieu of expressed genes inside the cells. In DLBCL, abnormal down-regulation of the tumor suppressor *PRDM1* due to an overexpressed let-7 may represent one of the mechanisms by which the latter promotes lymphomagenesis. Moreover, let-7 is shown to regulate components of the translation machinery.[42] Global changes in translation may contribute to lymphoma development by altering levels of critical oncogenes and tumor suppressors. Moreover, let-7 has the capacity to down-regulate DICER, which mediates processing of pre-miRNA to mature miRNA in the cytoplasm.[43] Thus, let-7 overexpression may affect the steady state levels of many other miRNAs with tumorigenic potentials. Interestingly, while let-7a, let-7b, and let-7f are all over-expressed in primary DLBCL cases, only let-7b is over-expressed in some DLBCL cell lines relative to GCB. The difference in let-7 expression between *in vivo* lymphomas and cultured lymphoma cells are in line with the previous observations that DLBCL cell lines are not faithful replica for primary DLBCL with respect to miRNA expression profiles.

By measuring endogenous PRDM1 changes on let-7 transfections in U2932 cells, we demonstrated directly a role of let-7 in modulating *PRDM1* expression in DLBCL. Though the modulatory effects of let-7 appear to be modest, it might have been underestimated owing to the limitations on the transfection efficiency (about 50%) and the artificial conditions of the experiments. The degree of suppression in *PRMD1* expression observed in this experiment may also be influenced by the half-life of the protein. Furthermore, the levels of PRDM1 in DLBCL are possibly modulated by additional inputs besides let-7. This is suggested by the observation that despite markedly higher levels of let-7b in the SUDHL-2 and U2932 cell lines compared with U266 (see Figure 3C), only SUDHL2 demonstrates a reduction in PRDM1 expression. For example, miRNAs other than let-7 may play a role in down-regulating *PRDM1*. Several other miRNAs are predicted to have potential binding sites in the 3′ UTR of the *PRDM1* gene, though only two of them, let-7 and miR-30, are among the most highly expressed miRNAs in DLBCL based on direct cloning. It is conceivable that besides let-7, miR-30 may also target *PRDM1* in DLBCL. MiR-30 has been shown to target *PRDM1* 3′ UTR by luciferase assays and has been postulated to regulate *PRDM1* expression during germinal center reaction.[36,38,44] Mechanisms other than miRNA, for example, those re-

lated to protein degradation, might also contribute to *PRDM1* posttranscriptional down-regulation. The steady state of PRDM1 in the cells may depend on an integrated summation of these inputs as well as the steady state levels of *PRDM1* transcripts. It is interesting that higher levels of *PRDM1α* mRNA are found in DLBCL harboring *PRDM1* inactivating mutations compared with those without such mutations. Although this observation may simply be attributed to a loss of the negative feedback signals due to total absence of PRDM1,[45] the possibility that stronger *PRDM1* induction signals are received by these cells before inactivating gene mutation events cannot be excluded. If the later scenario is indeed true, mutational inactivation rather than miRNA alone may be necessary to down-regulate PRDM1 to levels that promote outgrowth of lymphoma cells owing to the higher levels of *PRDM1* transcripts.

In summary, we show that protein levels of the tumor suppressor gene *PRDM1* may be down-regulated in DLBCL by miRNA-mediated down-regulation, in addition to mutational inactivation and transcription repression. MiRNA-mediated down-regulation of *PRDM1* as a potential pathogenetic event in lymphomagenesis has also been implicated in Hodgkin lymphoma[12] and EBV-positive Burkitt lymphoma.[46] Because B cell terminal differentiation is characterized by quantitative changes in PRDM1 levels,[47] its down-regulation may prevent it from reaching a threshold for terminal differentiation to proceed. Our studies provide supporting data on the importance of abnormal down-regulation of *PRDM1* and the consequent impairment of terminal B cell differentiation in the pathogenesis and/or maintenance of DLBCL.

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