Differential epigenetic regulation between the alternative promoters, PRDM1α and PRDM1β, of the tumour suppressor gene PRDM1 in human multiple myeloma cells

Raquel Romero-García1,2, Laura Gómez-Jaramillo1,2, Rosa María Mateos2,3, Gema Jiménez-Gómez1,2, Nuria Pedreño-Horrillo5, Esther Foncubierta1, Juan Francisco Rodríguez-Gutiérrez2,4, Sebastián Garzón2, A, Francisco Mora-López2,5, Carmen Rodríguez2,5, Luis M. Valor1,2 & Antonio Campos-Caro1,2,6*

Multiple myeloma (MM) is a B-cell neoplasm that is characterized by the accumulation of malignant plasma cells in the bone marrow. The transcription factor PRDM1 is a master regulator of plasma cell development and is considered to be an oncosuppressor in several lymphoid neoplasms. The PRDM1β isoform is an alternative promoter of the PRDM1 gene that may interfere with the normal role of the PRDM1α isoform. To explain the induction of the PRDM1β isoform in MM and to offer potential therapeutic strategies to modulate its expression, we characterized the cis regulatory elements and epigenetic status of its promoter. We observed unexpected patterns of hypermethylation and hypomethylation at the PRDM1α and PRDM1β promoters, respectively, and prominent H3K4me1 and H3K9me2 enrichment at the PRDM1β promoter in non-expressing cell lines compared to PRDM1β-expressing cell lines. After treatment with drugs that inhibit DNA methylation, we were able to modify the activity of the PRDM1β promoter but not that of the PRDM1α promoter. Epigenetic drugs may offer the ability to control the expression of the PRDM1α/PRDM1β promoters as components of novel therapeutic approaches.

PRDM1 (PR domain zinc finger 1) is a transcriptional factor that was initially identified as a 98-kDa DNA-binding repressor of β-interferon gene expression1, which is broadly known as BLIMP1 (B lymphocyte-induced maturation protein-1) because of its key role in the terminal differentiation of B cells towards plasma cells (PCs)2. Moreover, PRDM1 expression is required for the formation of Ig-secreting cells3 and the maintenance of long-lived PCs in the bone marrow4. However, PRDM1 has been subsequently shown to play critical regulatory roles in the differentiation, activation and homeostasis of other cell types5,6, development-related pathways in embryos and homeostasis in adults5,8.

The N-terminal half of the protein contains the PR domain, a subclass of the Su(var)39-1, Enhancer of Zeste, Trithorax (SET) family of methyltransferase domains that methylate histone H3 on lysine 9 (H3K9)9, which
is a feature of the proteins belonging to this family. However, in contrast to other members of this family, no methyltransferase activity has been demonstrated by PRDM1. Therefore, PRDM1 achieves its repressor activity by acting as a corepressor with other proteins, including histone deacetylases12, 9xa lysine methyltransferase13, protein arginine methyltransferase 5 (PRMT5)14, lysine-specific demethylase 1 (LDS1)15 and Groucho family proteins16. PRDM1 has been proposed to have modular control of gene expression17 with the ability to regulate different subsets of genes depending on the domains and proteins with which it interacts18.

The human PRDM1 gene contains seven coding exons and presents two alternative promoters capable of generating the two transcript isoforms: PRDM1α (NM_001198) and PRDM1β (AY198415). PRDM1β is generated through a promoter and an additional exon (exon-1β) located in intron 3 adjacent to exon 4. Exon-1β contains a short 5′ UTR and encodes only 3 aa (MEK) before joining exons 4 to 7. Therefore, PRDM1β is a shorter isoform (691 aa) than PRDM1α (825 aa), lacking 134 aa of the N-terminus (which comprises a small acidic region and a portion of the PR regulatory domain in PRDM1α). The PRDM1β isoform exhibits significantly impaired repressor activity in multiple target genes14, similar to PR-related isoforms of other PRDMs, such as PRDM217, PRDM318 and PRDM1619. The presence of the shorter isoform with a hypomorphic function may result in an imbalance in a “yin-yang” fashion between the two isoforms and may be critical for tumorigenesis20. This PRDM1α/PRDM1β imbalance can be the result of inactivating PRDM1α by means of gene mutations21–23 or promoter hypermethylation24,25 and by the activation/overexpression of PRDM1β26,27. In the latter case, an increase in the PRDM1β isoform at the mRNA level has been detected in both myeloma cell-derived lines and multiple myeloma samples6,28 and in lymphomas (diffuse large B-cell lymphoma30,31, T-cell lymphoma30 and EBV-associated lymphomas32,33). While the PRDM1α promoter has been extensively studied in mice34–36 and humans37–39, few studies have examined the PRDM1β promoter, and they were focused on its methylation status in lymphomas30–37. To date, there are no reports of its role in multiple myeloma.

Moreover, we demonstrated that PRDM1β isoform expression is augmented in multiple myeloma cells isolated from patient samples38, which was confirmed and correlated with the disease status of myeloma patients in a subsequent study38. However, the effect of PRDM1α and/or PRDM1β overexpression on proliferation and apoptosis has not been previously tested in myeloma cells. To this end, U266, NCI-H929 and RPMI-8226 cells were transfected with vectors expressing the PRDM1α and PRDM1β isoforms. Figure 1A shows that the overexpression of PRDM1α, but not PRDM1β, increased the apoptosis of the U266-transfected cells. However, neither PRDM1α nor PRDM1β overexpression affected the proliferation rate (Fig. 1B). Considering these observations and because it is virtually impossible to design specific knockdown assays for both isoforms, as their cDNA coding sequence only differs in 3 codons, we reasoned that the lower PRDM1α/PRDM1β ratio in myeloma cells, compared to that in normal cells, caused the accumulation of malignant cells due to inhibited apoptosis, not an increase in cell proliferation. More sophisticated manipulations would determine whether this reduction in apoptotic events contributes to the development of the myeloma. Therefore, we decided to analyse the unexplored transcriptional regulation of the PRDM1β isoform and the effect of epigenetic regulators on the expression of both isoforms in myeloma cells.

**Results**

**Overexpression of PRDM1α but not PRDM1β induces the apoptosis of U266 cells.** Impaired PRDM1β ability to repress important genes implicated in the cell cycle (e.g., the MYC gene) has been described16. Moreover, we demonstrated that PRDM1β isoform expression is augmented in multiple myeloma cells isolated from patient samples38, which was confirmed and correlated with the disease status of myeloma patients in a subsequent study38. However, the effect of PRDM1α and/or PRDM1β overexpression on proliferation and apoptosis has not been previously tested in myeloma cells. To this end, U266, NCI-H929 and RPMI-8226 cells were transfected with vectors expressing the PRDM1α and PRDM1β isoforms. Figure 1A shows that the overexpression of PRDM1α, but not PRDM1β, increased the apoptosis of the U266-transfected cells. However, neither PRDM1α nor PRDM1β overexpression affected the proliferation rate (Fig. 1B). Considering these observations and because it is virtually impossible to design specific knockdown assays for both isoforms, as their cDNA coding sequence only differs in 3 codons, we reasoned that the lower PRDM1α/PRDM1β ratio in myeloma cells, compared to that in normal cells, caused the accumulation of malignant cells due to inhibited apoptosis, not an increase in cell proliferation. More sophisticated manipulations would determine whether this reduction in apoptotic events contributes to the development of the myeloma. Therefore, we decided to analyse the unexplored transcriptional regulation of the PRDM1β isoform and the effect of epigenetic regulators on the expression of both isoforms in myeloma cells.

**Identification of a repressive regulatory element of PRDM1β expression.** To understand the regulatory mechanisms of transcriptional regulation for both isoforms and considering that the upstream regulatory elements and factors controlling the transcription of the human PRDM1β promoter have not been previously described, we decided to identify the putative cis regulatory elements that are critical for the basal expression of PRDM1β. To this end, we cloned and analysed a genomic fragment ~ 2 Kbp upstream of the transcription start site (TSS) in the PRDM1 gene extracted from GenBank, sequence accession number AL022067 (Fig. 2A). To evaluate the transcriptional mechanisms, the fragment was gradually truncated to create a series of 5′-end-deletion constructs cloned from positions − 2077 to + 41 (pGL3-BamHI), − 1343 to + 41 (pGL3-XbaI), − 678 to + 41 (pGL3-KpnI) − 420 to + 41 (pGL3-S1), − 304 to + 41 (pGL3-S2), − 124 to + 41 (pGL3-S3), and − 49 to + 41 (pGL3-S4) (numbered from the TSS according to the sequence AY19841516). All inserts were cloned upstream of the firefly luciferase reporter gene in a pGL3-basic vector (Fig. 2B). These constructs were transiently transfected into U266 myeloma cells, which expresses endogenous PRDM16,28. As shown in Fig. 2B, the most striking finding was the complete loss of promoter activity observed with the pGL3-S1 construct but not with the pGL3-S2 construct. The transcriptional recovery observed by the removal of the segment − 420/− 304 suggested that a cis-element with repressive actions on PRDM1β was present in this region (Fig. 2B). Similar results were obtained using the PRDM1β-expressing NCI-H929 cell line (Supplementary Fig. S1). RPMI-8226 was not used in this set of experiments because, as explained above, it did not express the PRDM1β isoform. After a sequence analysis of the S1-S2 fragments using Alibaba2 software based on the TRANSFAC database39, we identified several motifs as potential candidates for cis-acting elements (Supplementary Fig. S2). In addition to general transcription factors such as GATA, SP1, and OCT1, we observed a possible binding site for the transcription factor FOXA1 (also known as HNF3A), which is implicated in the establishment of lineage-specific transcriptional enhancers and programmes40. However, this DNA motif is shared among the FOX family members, and therefore,
we referred to this sequence indistinctly as the FOX or FOXA1 motif hereafter. Furthermore, this binding site matched one of the two half-sites in the consensus sequence for TP53 binding previously described by another group41 (Fig. 2A). Therefore, we decided to mutate this DNA motif as shown in Fig. 2C. Using luciferase assays, we confirmed the repressive role of this element, as the corresponding mutated FOXA1-site construct, which is homologous to the pGL3-S1 construct (pGL3-S1mutFOXA1), recovered the promoter activity to a level similar to that of the pGL3-S2 and pGL3-S3 constructs (Fig. 2B).

**Repressive elements of the PRDM1β promoter do not bind TP53.** Next, we further explored the potential role of the FOX-binding site in PRDM1β gene regulation. Thus, we prepared two probes from different regions of the S1–S2 construct that contained either the putative wild-type FOX-binding site (wtFOXA1) or the mutated binding site (mutFOXA1) (see sequences in Fig. 3A). The labelled probes were used in EMSAs with nuclear extract (NE) from U266 cells. As shown in Fig. 3B, different retarded band patterns were observed for the wtFOXA1 (lanes 1–2) and mutFOXA1 (lanes 7–8) probes, indicating that one or more factors were able to bind the wild-type but not the mutated sequences. Then, we investigated whether some of these bands were retarded because of TP53 binding to the wild-type probe. Thus, competition assays were conducted using wtFOXA1 (lane 3), mutFOXA1 (lane 4) and a double-stranded oligonucleotide containing the consensus binding site of TP53 (lane 5) as cold fragments (Fig. 3A). As expected, the retarded bands were abrogated with the wild-type version of the competitor probe, whereas the mutFOXA1 and TP53 cold fragments were unable to effectively compete with the labelled probe, demonstrating the specificity of the protein binding to the FOXA1 motif; in addition, the unidentified factor did not bind the putative TP53-binding site. This negative result was confirmed in a supershift experiment using two different antibodies against human TP53 (Fig. 3A, lane 6 and Supplementary Fig. S3), in which no changes in the band pattern were observed. Other related proteins (TP63 and TP73) were not detected (data not shown). Moreover, the anti-FOXA1 antibody did not lead to any supershift or eliminated retarded bands (Supplementary Fig. S3), indicating that these bands did not contain any of these transcription factors. To further corroborate that the retarded band corresponded to a factor other than the TP53 protein, additional EMSAs were performed using the wtFOXA1 and TP53 probes with U266 NE and recombinant TP53 protein. Figure 3C shows the different patterns of the bands obtained with the U266 NE and with recombinant TP53 using the wtFOXA1 probe (lane 1 vs. 3). After adding an anti-TP53 antibody to the
supershift assays, only the retarded band corresponding to TP53 was faded (lane 2 vs. 4). Under the same experimental conditions using a putative TP53 probe, the same retarded band was observed regardless of whether U266 NE or recombinant TP53 was used (lanes 5–8). This assay also confirmed the expression of endogenous TP53 in the U266 cells. These results indicated that the retarded band observed with the wtFOXA1 probe was not a consequence of TP53 binding to the probe. Furthermore, HeLa cells, which do not express TP53, were transfected with constructs containing the human TP53 cDNA, in either the wild-type form (wtTP53) or a mutated dominant negative form (TP53DN), to examine their effects on the regulation of the PRDM1α promoters. Thus, the PRDM1α and PRDM1β transcript levels in the HeLa cells transfected with wtTP53, TP53DN or an empty vector were analysed by RT-qPCR, and no difference was observed between them (Supplementary Fig. S4A), suggesting that, at least in this cell line, TP53 did not regulate PRDM1α gene transcription. Exogenous TP53 and TP53DN protein expression was confirmed by western blotting (Supplementary Fig. S4B). Therefore, in contrast to what has been described in cell line-derived colon cancer41, TP53 does not seem to have a role in PRDM1α transcriptional regulation. Future proteomic approaches should be applied to determine the identity of the regulatory factor that binds the repressive cis-element of the PRDM1β promoter.

Promoter methylation status shows a higher correlation with PRDM1β expression than with PRDM1α expression. In a previous report, we showed that the expression level ratio of PRDM1β/PRDM1α was altered in plasma cells from bone marrow samples of multiple myeloma patients28, Due to the relevant role of epigenetics in cancer and tumorigenesis, we investigated DNA methylation patterns in a more natural environment (as opposed to the in vitro assays described above) in our cellular preparations. To correlate their respective expression levels to the methylation status of their promoters, we examined the methylation pattern in both promoters within the same samples isolated from several cell lines and primary cells from healthy donors and patients (naïve B-cells, PCs and MM-PCs). In total, 35 and 12 CpG dinucleotides in the PRDM1α and PRDM1β promoters, respectively, were selected as potentially methylable for bisulfite sequencing analysis (Fig. 4A). The PRDM1α promoter was hypermethylated compared to the PRDM1β promoter in all tested cell types. Thus, cells well known to be negative for PRDM1α expression, such as the Raji and Daudi lymphoma cell lines, and normal B cells, showed high methylation levels, ~68%, ~77% and ~89%, respectively (Fig. 4A). Strik-
Figure 3. The repressive element of the human PRDM1β promoter binds nuclear proteins but not FOXA1 or TP53. 

a) Sequences used as probes or cold competitor fragments (only the DNA strand + is represented). Putative wtFOXA1 and TP53-binding sites are indicated. Sequences in the mutated FOXA1 fragment and a sequence used as a putative TP53 (Panomics, Fremont, CA) are also shown.

b) EMSA with NE from the U266 cell line and wtFOXA1 or mutFOXA1 probes. Lane 1, probe without NE; lane 2, probe incubated with NE; lanes 3, 4 and 5 correspond to the competition experiments using cold fragments (100× excess) with the same sequence as the labelled probe (wtFOXA1), the mutated version (mutFOXA1) or the TP53 binding site fragment, respectively; lane 6, supershift assay performed using the same conditions as indicated for lane 2 with the addition of an anti-human TP53 specific antibody (Sigma, P6874); lanes 7 and 8, mutated probe FOXA1 without or with U266 NE, respectively. The main retarded band is indicated by an arrow.

c) Lanes 1 and 3, EMSA using a wtFOXA1 probe with U266 NE showing the positions of the main retarded band (indicated by an arrow) and the shifted band observed using recombinant human TP53 protein instead of NE (indicated by an arrowhead). Lanes 2 and 4 show the same conditions as lanes 1 and 3 with the addition of an anti-TP53 antibody for the supershift assays. Lanes 5–8, EMSAs and similar to lanes 1–4 but with the TP53 probe instead of the FOXA1 probe.
a) 

![Diagram of PRDM1α and PRDM1β CpG regions with methylation levels in different cell lines and donor cells.]

b) 

![Bar graphs showing average % methylation for PRDM1α and PRDM1β across different CpG sites in various cell lines and donor cells.]

c) 

![Graph showing relative mRNA levels of PRDM1α and PRDM1β in Daudi, Raji, U266, NCI-H929, and RPMI-8226 cell lines. Western blot images of PRDM1α and PRDM1β proteins in U266, NCI, RPMI, Daudi, and Raji cell lines with molecular weights indicated.]
The transcriptional activity of PRDM1 is essential for the differentiation and maintenance of long-lived plasma cells, whose downregulation has been associated with several types of cancer. Therefore, PRDM1 is considered a tumour suppressor gene\textsuperscript{23,43–47}. The two major isoforms, PRDM1α and PRDM1β, are expressed in myeloma cells, while only the PRDM1α isoform is expressed in non-tumorigenic plasma cells\textsuperscript{11,28}. PRDM1β is a truncated
isoform that is thought to compete with the full-length isoform PRDM1α, which is overexpressed in myeloma cells, leading to the hypothesis that PRDM1β may be functionally relevant in myeloma cells. Nevertheless, there are few studies linking the expression of PRDM1β to myeloma. In this study, we tested, for the first time, the effect of the overexpression of PRDM1α and PRDM1β isoforms in myeloma cells. We observed that PRDM1α, but not PRDM1β, induced increased apoptosis without apparently affecting cell proliferation in myeloma. These observations led us to consider an alternative explanation for the maintenance of myeloma cells: Rather than an increase in proliferation, expression of the PRDM1β isoform may inhibit apoptosis. However, some contradictory results were obtained in other studies in which PRDM1α altered the proliferation of HCT116 and SW620 cells but not RKO colon cancer cells, and ectopic expression of PRDM1 in a chicken fibroblast cell line inhibited cell proliferation. Depending on the cellular context, further studies on the mechanisms of action of the PRDM1

Figure 5. PRDM1α and PRDM1β expression after 5-aza-dC treatment. a Schematic probe and primer pair locations on cDNA used for detecting the two transcripts (the sequences are listed in Table 1). b MM-PCs and RPMI-8226, NCI-H929 and U266 cells were treated with the DNA methyltransferase inhibitor 5-aza-dC (0, 0.5, 1 and 2 µM) for 2 days. RT-qPCR assays of PRDM1α and PRDM1β transcript expression were performed. The mean ± SEM of n = 3 independent experiments are shown. (*) Indicates p < 0.05.
Figure 6. Histone marks at the PRDM1α and PRDM1β promoters in U266 and RPMI-8226 cells. ChIP assays of two MM cell lines, i.e., U266 (positive for PRDM1α and PRDM1β) and RPMI-8226 (positive only for PRDM1α), using antibodies against mono- or tri-methylated histone H3-Lys4 (H3K4me1 or H3K4me3) or di-methylated or acetylated H3-Lys9 (H3K9me2 or H3K9ac). The samples were analysed by RT-qPCR. The relative levels of enrichment compared to the controls are shown as the means ± SEM; n = 4 independent experiments. (*) Indicates p < 0.05. Normal rabbit serum was used as a negative control (C-).
isoforms will be important for understanding their roles in the regulation of apoptosis, cell proliferation and the cell cycle.

In the present study, luciferase promoter analysis identified a putative cis-element in the promoter region -304 to -420 relative to the TSS. The in silico analysis identified a possible binding site for the FOX transcription factors in positions -345 to -334 within that region. Moreover, this site partially overlapped with a previously proposed DNA binding motif for TP5341, in the previous work, the authors described a transcriptional regulation loop through which PRDM1 is activated by TP53, which is then repressed by PRDM1. However, we were unable to reproduce TP53 involvement in the regulation of PRDM1 in the EMSAs, an in vitro assay in which proteins bind DNA motifs solely by sequence affinity, or in the overexpression experiments, in which we did not observe any increase/decrease in PRDM1α or PRDM1β levels in HeLa or U266 cell cultures with western blotting or RT-qPCR (Supplementary Fig. S3 and data not shown, respectively). This lack of reproducibility may be due to, at least, two reasons: (1) the PRDM1/TP53 autoregulatory loop may be dependent on the cell line studied (HTC116 cells in the case of the original publication)41, and (2) the previous study referred to PRDM1 activation or repression without clearly distinguishing between the two isoforms PRDM1α and PRDM1β, despite the identification of the TP53 binding site in the PRDM1β promoter. However, we failed to identify a transcription factor bound to this promoter in the supershift assays (TP53, Fig. 3 and Supplementary Fig. S2) or in a preliminary mass spectrometry analysis (not shown).

In addition, the previous experiments were based on exogenous DNA sequences that did not properly reproduce the cellular chromatin context. Here, we report, for the first time, the methylation status of alternative promoters of the PRDM1 gene from isolated myeloma cells. We found that the PRDM1α promoter was relatively hypermethylated (range 40–90%) in all the cell lines tested regardless of the expression of the PRDM1α transcript or protein. However, the PRDM1β promoter generally presented with a much lower methylation level (10–25%) with significant differences in the methylation of specific CpG positions in the cells expressing and not expressing PRDM1β. CpG-5, CpG-9 and CpG-12 clearly had greater methylation enrichment in the PRDM1β-negative cells than in PRDM1β-positive cells. No difference in the methylation of any specific CpG position was observed in the PRDM1α promoter. In summary, the following conclusions are drawn based on our methylation analysis. First, the overall level of methylation at the PRDM1α promoter does not determine its expression in all cells tested; for instance, the Rai cell line (~68% methylated) does not express PRDM1α, while the NCI-H929 cell line (~68%) and MM cells (~74%) both highly express PRDM1α. Second, even though the global methylation level of the PRDM1β promoter (~25%) is much lower than that of the PRDM1α promoter (~40%), the methylation level does not appear to be decisive of the relative expression levels of both isoforms, although one or both promoters may be activated by 5-aza-dC (this article and previous works42,43,47). In addition to methylation, other elements, such as specific transcription factors and miRNAs, may be critical for the imbalance expression of PRDM1α/PRDM1β in tumors48,49. Nevertheless, differing results of the methylation status of the PRDM1α promoter have been reported. The hypermethylation of the PRDM1α promoter in EBV+ BL cell lines, including the Daudi cell line, which is hypermethylated as described in Fig. 4A, is associated with low PRDM1 expression50. Nonetheless, in contrast to our work, the authors observed only trace or no methylation in normal B-cell subsets (i.e., GC cells and memory and naive B cells), although it is widely reported that these cell subsets do not express PRDM1α, which is a marker of differentiated PCs. In other reports, the low PRDM1α transcript levels were not the result of promoter hypermethylation in diffuse large B-cell lymphoma51 or chronic lymphocytic leukaemia52, but the opposite results have also been reported in NK-cell malignancies53. In conclusion, the general lack of consistency between promoter methylation and the gene expression of PRDM1α in previous works does not support a relevant role for CpG methylation in the regulation of this isoform. This conclusion is supported by the results of the cell culture treatment with the methyltransferase inhibitor 5-aza-dC, which significantly increased the expression of PRDM1β but not PRDM1α (Fig. 5).

Another important finding in this study is the differential posttranslational modifications in the histones at PRDM1 promoters in the PRDM1β-expressing and non-expressing cell lines. Indicative of promoter activation, similar levels of H3K4me3 and H3K9ac were found in both promoters in U266 cells (PRDM1α positive and PRDM1β positive) and RPMI-8226 cells (PRDM1α positive and PRDM1β negative). However, the enrichment of H3K4me1, H3K9me2, and, to a lesser extent, H3K27me3 was much higher at the PRDM1β promoter in the RPMI-8226 cells, suggesting that the abundance of these modifications may regulate the transcriptional activity of the PRDM1β promoter towards repression. In general, the H3K4me1 histone mark has been associated with enhancer activity, particularly in B-cell fate54, and it may also be possible that the PRDM1β promoter acts as an enhancer/promoter region “poised” to be activated while awaiting additional signalling (e.g., acetylation of H3K9).

PRDM1 expression has been considered an oncogene in MM55, but in-depth analysis of the differential expression of its isoforms has been lacking, until the present work was performed. We and other researchers have analysed public microarray data comparing normal plasma cells and samples from multiple myeloma, and no correlations were observed for low or high PRDM1 expression levels with the prognosis of the disease46. Unfortunately, these analyses were mainly based on microarray data, which were not designed to discriminate between PRDM1α or PRDM1β isoforms, and new analysis data sets (RNA-seq, new microarrays, etc.) comprising possible alternative isoforms are necessary to discern whether differential PRDM1 expression may be related to the pathophysiology of the disease. In conjunction with the transcriptional regulation described in this study, other post-transcriptional regulatory mechanisms involving miRNAs may be key to regulating the expression of PRDM1, as altered expression of some miRNAs targeting PRDM1 mRNA has been described during plasma cell differentiation and in malignant plasma cells55,57. Whether these miRNAs specifically modulate the PRDM1α or the PRDM1β isoform remains to be explored.

In summary, our results indicated that PRDM1 gene promoters are modulated through a broad range of genetic and epigenetic control mechanisms that depend on the tumour cell line, supporting further investigation...
into the transcription and epigenetic factors involved in the direct or indirect regulation of PRDM1β transcription and their possible implications in myelomagenesis. In the case of novel treatments of multiple myeloma (Fig. 7), the different transcriptional activity found in the PRDM1α and PRDM1β promoters in response to treatment with epigenetic modifiers may pave the way to the generation of more specific and tailored epigenetic-based therapeutic strategies for this disease by favouring the expression of PRDM1α over that of PRDM1β.

**Materials and methods**

**Human cell lines.** The human cell lines U266, NCI-H929, RPMI-8226, HeLa, Daudi and Raji were obtained from The European Collection of Authenticated Cell Cultures (ECACC, Sigma-Aldrich, Madrid, Spain) and maintained in RPMI 1640 medium (Gibco Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco) at 37 °C in a humidified atmosphere containing 5% CO2 and were routinely verified by morphology and periodically checked for Mycoplasma contamination.

**Patient tissue samples and normal cells.** Multiple myeloma PCs (MM-PCs) were purified from donated bone marrow aspirates from patients diagnosed with MM (excluding MGUS or SMM) using monoclonal antibody anti-CD138-coated microbeads (Miltenyi-Biotec, Auburn, CA, USA). Normal PCs were obtained from discarded bone marrow specimens with no abnormalities at diagnosis after FAC sorting CD138− CD19++ CD38+++ cells. Naïve human B-lymphocytes were isolated from peripheral blood samples from normal volunteers after T-cell depletion by a rosette technique and FAC-sorted to obtain CD19+ CD27− IgD+ cells. All human samples (MM n = 7, normal PCs, n = 4, and B-cells, n = 3) were obtained from adult donors upon written informed consent under the supervision and approval of the Institutional Review Board (code PI11/01091, date 11/10/2011, Ethics Committee for Research of the Hospital Universitario “Puerta del Mar”, Cadiz, Spain). The Andalusian Public Health System Biobank coordinated the collection, processing, management and assignment of all biological samples used in this study according to standard procedures established for this purpose.

**Reagents.** Antibodies were purchased from the following suppliers: anti-H3K9me2, anti-H3K27me3 and anti-H3K9ac (EpiGentek, Farmingdale, NY); anti-H3K4me3, anti-H3K4me1 and anti-β-actin (Abcam, Cambridge, UK); negative rabbit IgG (Cell Signaling Technologies); anti-PRDM1 (sc-66015, Santa Cruz Biotechnol-
ogies, Dallas, TX); anti-TP53 (P6874 from Sigma and sc-98 from Santa Cruz); anti-mouse HRP-conjugated and anti-tubulin (Sigma); and anti-FOXA1 from Santa Cruz. Recombinant human TP53 protein (ref. 506165) was obtained from Calbiochem (San Diego, CA). The inhibitor 5-aza-2′-deoxycytidine (5-aza-dC) was purchased from Sigma-Aldrich and dissolved in DMSO.

Isolation of the 5′ flanking region of the human PRDM1β promoter and luciferase reporter assays. The 5′-flanking region of the human PRDM1 gene (~2077/+41) was isolated by PCR. Two specific primers, PRDM1β-BamHI-F (forward, ~2077 bp relative to the translation start site including a restriction site for the BamHI enzyme) and PRDM1β-BglII-R (reverse, +41 bp relative to the translation start site including a part of exon-1β and a restriction site for the BglII enzyme) (Table 1), were selected on the basis of the sequence of the human PRDM1 genomic DNA accession number AL022067. The resulting PCR fragment was digested with BamHI/BglII and cloned into a pGL3-basic firefly luciferase reporter vector (Promega, Madison, WI) at the BglII site to obtain the pGL3-BamHI construct. This construct was digested with the NheI/XbaI restriction enzymes to obtain the pGL3-XbaI construct, which was subsequently digested with the KpnI restriction enzyme to obtain the pGL3-KpnI construct. The pGL3-S1, pGL3-S2, pGL3-S3 and pGL3-S4 constructs were generated by PCR using the following forward oligonucleotide primers, respectively, in Table 1: PRDM1β-S1-F, PRDM1β-S2-F, PRDM1β-S3-F, and PRDM1β-S4-F.

Table 1. Oligonucleotide sequences used for the different assays. All sequences are shown from 5′ → 3′. Lowercase letters indicate endonuclease restriction sites just intended for cloning, or just in case of probe mutFOXA1 for EMSA indicate the nucleotides mutated. FAM is fluorescein amidite fluorophore; ZEN is IDT’s ZEN dark quencher; IABFQ is IDT’s Iowa Black FQ dark quencher.

| Cloning reporter constructs       |       |
|----------------------------------|-------|
| PRDM1β-BamHI F                   | ctcgagattccGTGCAATCTTACATGAAATG |
| PRDM1β-S1 F                      | tccpgGTGTTTTAGCCAGCCTCC         |
| PRDM1β-S2 F                      | tccggGTCAAATTGCGGCTG           |
| PRDM1β-S3 F                      | tccggGTAGAAACGCCGAGTACAG        |
| PRDM1β-S4 F                      | tccggCTACCCAGTCTCAAAAGCAC       |
| PRDM1β-BglII R                   | tctagatTTTGTACTGAGTAATG         |

| ChIP-qPCR                        |       |
|----------------------------------|-------|
| PRDM1α F                         | GGAAGCCAGAGGTTAAC                  |
| PRDM1α R                         | CTGGGCGCTCCTCCTG                    |
| PRDM1β F                         | GTTGGAAACGCCGAGTACAG               |
| PRDM1β R                         | AGATCTTTTCCATCTAGTTTACGTTTCG       |

| Bisulfite sequencing nested PCR   |       |
|----------------------------------|-------|
| PRDM1β 1F                        | GGTGTTTATATGATAATAGTTATTTTAGG     |
| PRDM1β 1R                        | CCTACTACCTAATGAAAAAATGCACATC      |
| PRDM1β 2F                        | TGGGGGGTGAGGATGTTTTGTATAGTG       |
| PRDM1β 2R                        | ATCCAAACATTTGACAGAAT             |
| PRDM1α 1F                        | AGAATATAATAGTTAATGAAGAGTG         |
| PRDM1α 1R                        | AAATTTCTAACTATTTTCAAATTA          |
| PRDM1α 2F                        | AAGTTTTTTAAGAGGAGTGAAGAGG         |
| PRDM1α 2R                        | CCAAAAATATCCACACTATTTAAAACTCTC    |

| Transcripts expression by RT-qPCR |       |
|----------------------------------|-------|
| PRDM1α exon 3-4                  |       |
| Probe                            |       |
| Primer 1                         | FAM/TGGAGGATGC/TCATTCCAGAGG/GAC/IABFQ |
| Primer 2                         | AGATGTTGCTGAGTTCCAGATAG           |
| PRDM1β exon 1β-4                 |       |
| Probe                            |       |
| Primer 1                         | FAM/CGCCCTGAGA/ATAGGCTTTGACTCT/TCATIABFQ |
| Primer 2                         | TTGGAGGAGTTCACTTAATAG             |
| β-actin                          |       |
| Probe                            |       |
| Primer 1                         | FAM/TGATTCCATG/TCGAGTGGTGCGG/AATAG |
| Primer 2                         | CATTACGCGGTGAAAGTG                 |

| EMSA probes                      |       |
|----------------------------------|-------|
| wtFOXA1 strand+                  | GGGAAATGCGAATGCTGAGATGTTTATCAAGAGG |
| wtFOXA1 strand−                  | CTCCTGATAAACATGCTGAGCTTCAGCTTC   |
| mutFOXA1 strand+                 | GGAAGGCGAATGCTGAGCTACAGTCAAGAGG  |
| mutFOXA1 strand−                 | CTCCTGATAAGGCTGAGCTGAGCTTCAGCTTC |
| TP53 strand+                     | TACAGAAATGCTAAGAGTACGCTGGG       |
| TP53 strand−                     | GCCCCAGATGCTGAGACTATGTCGTA       |

Table 1. Oligonucleotide sequences used for the different assays. All sequences are shown from 5′ → 3′. Lowercase letters indicate endonuclease restriction sites just intended for cloning, or just in case of probe mutFOXA1 for EMSA indicate the nucleotides mutated. FAM is fluorescein amidite fluorophore; ZEN is IDT’s ZEN dark quencher; IABFQ is IDT’s Iowa Black FQ dark quencher.
S2-F, PRDM1β-S3-F and PRDM1β-S4-F (all including a restriction site for the enzyme SmaI at the 5’ ends) together with the PRDM1β-BglIII-R oligonucleotide as the reverse primer (Table 1). The resulting PCR products were digested with SmaI/BglIII restriction enzymes and cloned into a pGL3-basic plasmid. Primary sequences of the inserts were validated by Sanger sequencing. All PRDM1β promoter constructs were transfected into U266 cells using the Nucleofector Kit C Program X-005 with an Amaxa nucleofector system following the manufacturer’s instructions (Lonza, Barcelona, Spain) or into NCI-H929 cells using a Gene Pulser II apparatus (Bio-Rad, Madrid, Spain) set at 400 µF and 240 V, with a time constant of t ~ 20 ms. The cells were harvested after 48 h, and luciferase activity was measured using a dual luciferase reporter assay kit (Promega). Each transfection experiment was performed in triplicate with at least two different plasmid preparations.

**Chromatin immunoprecipitation assays.** The chromatin immunoprecipitation (ChiP) procedure was performed using a SimpleChiP Enzymatic Chromatin IP Kit as recommended in the manufacturer’s instructions (Cell Signaling Technologies, Barcelona, Spain). Briefly, RPMI-8226 cells or U266 cells (40 × 10⁶) were performed using a SimpleChIP Enzymatic Chromatin IP Kit as recommended in the manufacturer’s instructions (Cell Signaling Technologies, Barcelona, Spain). Purified using spin columns. Real-time PCR was performed with the corresponding specific primers indicated in Table 1.

Promoter methylation analysis. The methylation of the PRDM1α and PRDM1β promoters was assessed by bisulfite sequencing. Genomic DNA was extracted from all cell types analyzed using a ZR-Duet DNA/RNA Miniprep kit (Zymo Research, Irvine CA), and then, 500 ng of DNA was used for the bisulfite modification with a premium bisulfite kit according to the manufacturer’s instructions (Diagenode, Belgium). Bisulfite sequencing nested-PCR was performed to assess the methylation of the CpG islands in the PRDM1α and PRDM1β promoters using PRDM1α and PRDM1β bisulfite-specific primers (Table 1) and Q5 High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, MA). The amplified DNA was cloned into a pSpark I vector (Canvax, Córdoba, Spain), and more than 10 individual clones were Sanger sequenced. The obtained sequences were analysed by the online BISMA (bisulfite sequencing DNA methylation analysis) program.

5-Aza-dC treatment. RPMI-8226, U266, NCI-H929 or MM-isolated cells were cultured with or without the inhibitor 5-aza-2′-deoxycytidine (5-aza-dC; 0.5, 1, or 2 µM) (Sigma), which was initially dissolved in DMSO. The cells were cultured at 37 °C in a 5% CO₂ incubator for 48 h. After incubation, the viability of the cells was measured using Trypan blue, and the variations in the expression of the PRDM1α and PRDM1β promoters were assessed by RT-qPCR. All reactions were performed with identical conditions in triplicate.

Retrotranscription and RT-qPCR. For the PRDM1α and PRDM1β transcript analysis, the total RNA from the cultured cells was purified using NucleoSpin RNA XS (Macherey–Nagel, Düren, Germany) and reverse transcribed using random hexamers with a Maxima H Minus First Strand cDNA synthesis kit (Thermo Fisher, Waltham, MA). Then, the cDNAs were assessed with real-time PCR TaqMan probes for PRDM1α, PRDM1β and β-actin (Table 1) and a SensiFAST Probe No-ROX kit (Bioline, London, UK) in a RotorGene 6000 (Corbett, Sydney, Australia). The relative quantitative values were calculated using the 2^−ΔΔCT method.

Western blotting. Western blotting was performed previously described. Fifty micrograms of total protein lysates were added to each well. The following antibodies were used at the indicated dilutions: anti-PRDM1 (sc-66015 from Santa Cruz Biotechnologies, Dallas, TX), anti-β-actin (ab6276 from Abcam), anti-TP53 (P6874) and anti-mouse HRP-conjugated (A2554 from Sigma). The bound antibodies were detected using a Western-Bright Sirius kit (Advansta, Menlo Park, CA) and a ChemiDoc XRS system (Bio-Rad, Madrid, Spain). As indicated, the blots were reprobed with the β-actin or α-tubulin antibody as a loading control.

Electrophoretic mobility shift assays (EMSAs). Nuclear extracts (NEs) were prepared from the cells by hypotonic lysis, followed by high salt extraction of nuclei as previously described. All probe and competitor DNA fragments used for the competition assays (Table 1) were obtained by annealing synthesized complementary oligonucleotides with protruding ends and labelled by Klenow filling with [α-32P]-dCTP (Perkin-Elmer, Madrid, Spain). The DNA–protein-binding assays were carried out as previously described. For the competition studies, 100-fold molar excess competitor DNA was added to the reaction mixture 10 min prior to the labelled probe. Supershift assays were performed by preincubating the NEs with an antibody against TP53 purchased from Sigma (P6874) or Santa Cruz (sc-98) as indicated or anti-FOXa1 purchased from Santa Cruz (sc-6553) for 1 h on ice before the probe addition. As indicated, recombinant human TP53 protein (ref. 506165) from Calbiochem (San Diego, CA) was used instead of the nuclear extracts.
Apoptosis and cell proliferation assays. U266, RPMI-8226 and NCI-H929 cells were transfected with a pIRE2-EGFP empty vector (Clontech, Takara Bio USA Inc, USA) or a vector containing the cDNAs for PRDM1α or PRDM1β, both with an HA epitope. The cells were harvested after 48 h and treated for apoptosis or proliferation analysis. Apoptosis was determined by Annexin V-allophycocyanin (APC) staining according to the protocol of the manufacturer (ImmunoTools, Friesoythe, Germany). Cell proliferation was determined with a Click-IT plus Edu Alexa 647 flow cytometry assay kit following the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Only EGFP–transfected cells were selected for apoptosis and proliferation analyses by a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, Franklin Lakes, NJ). To validate the transfections, Western blotting was performed as described above using a conjugate HRP antibody against the HA epitope (ref. A00169, GenScript, Piscataway, NJ).

Statistical analysis. The statistical package SPSS version 21 was used. All variables exhibited a nonparametric distribution after applying the Kolmogorov–Smirnov test; thus, the data were analysed using the nonparametric Mann–Whitney U test (two sided) to determine significant differences between two experimental groups. The sample size (n) for each experimental group/condition is given in the corresponding figure legend. The results are generally expressed as the means ± SEM, and p < 0.05 was considered statistically significant.
bioinformatic analyses and contributed to result interpretation and figure preparation; J.F.R.-G. and S.G. pro-

R.R.-G., L.G.-J., R.M.M.-B., G.J.-G., N.P.-H., E.F., F.M.-L., C.R. and A.C.-C. carried out all experiments and

Consejería de Innovación, Ciencia y Empresa-Junta de Andalucía through Project P09-CTS-5445 to ACC, and

(30) Zhao, W. L. et al. PRDM1 is involved in chemoresistance of T-cell lymphoma and down-regulated by the proteasome inhibitor. *Blood* **111**, 3867–3871 (2008).

(31) Tunyaplin, C., Shapiro, M. A. & Calame, K. L. Characterization of the B lymphocyte-induced maturation protein-1 (Blimp-1) gene, mRNA isoforms and basal promoter. *Nucleic Acids Res.* **28**, 4846–4855 (2000).

(32) Morgan, M. A. et al. Blimp-1/Prdm1 alternative promoter usage during mouse development and plasma cell differentiation. *Mol. Cell. Biol.* **29**, 5813–5827 (2009).

(33) Tunyaplin, C. et al. Direct repression of prdm1 by Bcl-6 inhibits plasmacytic differentiation. *J. Immunol.* **173**, 1158–1165 (2004).

(34) Ochiai, K., Muto, A., Tanaka, H., Takahashi, S. & Igarashi, K. Regulation of the plasma cell transcription factor Blimp-1 gene by Bach2 and Bcl6. *Int. Immunol.* **20**, 453–460 (2008).

(35) Vasanwala, F. H., Kusam, S., Toney, L. M. & Dent, A. L. Repression of API-1 function: a mechanism for the regulation of Blimp-1 expression and B lymphocyte differentiation by the B cell lymphoma-6 protooncogene. *J. Immunol.* **169**, 1922–1929 (2002).

(36) Mora-Lopez, F., Pedreno-Herrillo, N., Delgado-Perez, L., Brieva, J. A. & Campos-Caro, A. Transcription of PRDM1, the master regulator for plasma cell differentiation, depends on an SP1/SP3/EGR-1 GC-box. *Eur. J. Immunol.* **38**, 2316–2324 (2008).

(37) Mora-Lopez, F., Reales, E., Brieva, J. A. & Campos-Caro, A. Human BSAP and BLIMP1 conform an autoregulatory feedback loop. *Blood* **110**, 3130–3137 (2007).

(38) Buda, G. et al. PRDI-BF1 and PRDI-BF1 isoform expressions correlate with disease status in multiple myeloma patients. *Hematol. Rep.* **9**, 7201 (2017).

(39) Grabe, N. AliBaba2: context specific identification of transcription factor binding sites. *Silico Biol.* **2**, S1–15 (2002).

(40) Lupien, M. et al. FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell* **132**, 958–970 (2008).

(41) Yan, J. et al. BLIMP1 regulates cell growth through repression of p53 transcription. *Proc. Natl. Acad. Sci. USA* **104**, 1841–1846 (2007).

(42) Cheng, J. et al. A role for H3K4 monomethylation in gene repression and partitioning of chromatin reads. *Mol. Cell** **53**, 979–992 (2014).

(43) Boi, M. et al. PRDM1/BLIMP1 is commonly inactivated in anaplastic large T-cell lymphoma. *Blood* **122**, 2683–2693 (2013).

(44) Hangaiashi, A. & Kurokawa, M. Blimp-1 is a tumor suppressor gene in lymphoid malignancies. *Int. J. Hematol.* **91**, 46–53 (2010).

(45) Liang, L. et al. The downregulation of PRDM1/Blimp-1 is associated with aberrant expression of miR-223 in extranodal NK/T-cell lymphoma, nasal type. *J. Exp. Clin. Cancer Res.* **33**, 7 (2014).

(46) Zhu, Z. et al. Downregulation of PRDM1 promotes cellular invasion and lung cancer metastasis. *Tumour Biol.* **39**, 1010428317695929 (2017).

(47) Kang, H. B. et al. PRDM1, a tumor-suppressor gene, is induced by genkwadaphnin in human colon cancer SW620 cells. *J. Cell. Biochem.* **117**, 172–179 (2016).

(48) Liu, C. et al. PRDM1 silences stem cell-related genes and inhibits proliferation of human colon tumor organoids. *Proc. Natl. Acad. Sci. USA* **115**, E5066–E5075 (2018).

(49) Wan, Z., Lu, Y., Rui, L., Yu, X. & Li, Z. PRDM1 overexpression induce GO/G1 arrest in DF-1 cell line. *Gene* **592**, 119–127 (2016).

(50) White, C. A. et al. Histone deacetylase inhibitors upregulate B cell microRNAs that silence AID and Blimp-1 expression for epigenetic modulation of antibody and autoantibody responses. *J. Immunol.* **193**, 5933–5950 (2014).

(51) Nie, K. et al. 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. *Am. J. Pathol.* **177**, 1470–1479 (2010).

(52) Duckworth, A., Glenn, M., Slupsky, J. R., Packham, G. & Kalakonda, N. Variable induction of PRDM1 and differentiation in chronic lymphocytic leukemia is associated with anergy. *Blood* **123**, 3277–3285 (2014).

(53) Iqbal, J. et al. Genomic analyses reveal global functional alterations that promote tumor growth and novel tumor suppressor genes in natural killer-cell malignancies. *Leukemia* **23**, 1139–1151 (2009).

(54) Lin, Y. C. et al. A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate. *Nat. Immunol.* **11**, 635–643 (2010).

(55) Alizart, M. et al. EZH2 inhibition in multiple myeloma downregulates myeloma associated oncogenes and upregulates microRNAs with potential tumor suppressor functions. *Oncotarget* **8**, 10213–10224 (2017).

(56) Valor, L. M., Rodriguez-Bayona, B., Ramos-Amaya, A. B., Brieva, J. A. & Campos-Caro, A. The transcriptional profiling of human in vivo-generated plasma cells identifies selective imbalances in monomeric gammaglobulins. *PLoS ONE* **12**, e0183264 (2017).

(57) Kassambara, A. et al. Global miRNA expression analysis identifies novel key regulators of plasma cell differentiation and malignant plasma cell. *Nucleic Acids Res.* **45**, 5639–5652 (2017).

(58) Rajkumar, S. V. et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol.* **15**, e538–548 (2014).

(59) Rohde, C., Zhang, Y., Reinhardt, R. & Jeltsch, A. RISMA—a fast and accurate bisulfite sequencing data analysis of individual clones from unique and repetitive sequences. *BMC Bioinform.* **11**, 230 (2010).

(60) Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402–408 (2001).

(61) Gomez-Jaramillo, L. et al. VAMP2 is implicated in the secretion of antibodies by human plasma cells and can be replaced by other synaptobrevins. *Cell. Mol. Immunol.* **15**, 353–366 (2018).

(62) Schreiber, E., Matthias, P., Muller, M. M. & Schafer, W. Rapid detection of octamer binding proteins with ‘mini-extracts’, prepared from a small number of cells. *Nucleic Acids Res.* **17**, 6419 (1989).

Acknowledgements

This research was funded by Instituto de Salud Carlos III through Projects PI11/01091 and PI15/01147 to ACC (Co-funded by the European Regional Development Fund/European Social Fund “Investing in your future”), Consejería de Innovación, Ciencia y Empresa-Junta de Andalucía through Project P09-CTS-5445 to ACC, and Consejería de Salud-Junta de Andalucía with Projects PI-0265/2005 and PI-0817/2012 to ACC and FML, respectively. LMV is the recipient of a Miguel Servet I contract (CP15/00180) from Instituto de Salud Carlos III and Fondo Social Europeo 2014-2020.

Author contributions

R.R.-G., L.G.-J., R.M.M.-B., G.J.-G., N.P.-H., E.F., F.M.-L., C.R. and A.C.-C. carried out all experiments and bioinformatic analyses and contributed to result interpretation and figure preparation; J.F.R.-G. and S.G. provided materials and data; L.M.V. contributed to result interpretation and figure preparation, review and editing; A.C.-C. conceived the study and supervised all experiments, funding acquisition, and wrote the manuscript.

Competing interests

The authors declare no competing interests.
