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# Transcriptional repression of plasma cell differentiation is orchestrated by aberrant over-expression of the ETS factor *SPIB* in Waldenström macroglobulinaemia

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## Summary

In Waldenström macroglobulinaemia (WM), the mechanism(s) responsible for repression of B-cell differentiation remains unknown. We found that expression of *SPIB* and *ID2* were significantly increased and decreased, respectively, in WM lymphoplasmacytic cells (LPC). Ectopic expression of *SPIB* in healthy donor $CD19^+$ cells inhibited plasmacytic differentiation in conjunction with decreased transcription of *IRF4* and *XBP1* spliced form. In primary WM LPC, knock-down of *SPIB* induced plasmacytic differentiation in conjunction with increased transcription of *PRDM1*, *XBP1* spliced form, *IRF4* and *ID2*. Knock-down of *SPIB* also led to decreased *BCL2* expression. Given that *SPIB* is a direct target of *POU2AF1* (*OBF1*) in complex with *POU2F2* or *POU2F1*, we next examined their expression in WM LPC. *POU2F2* transcription, as well as POU2F2 and POU2AF1 protein expression was higher in WM LPC. Ectopic expression of *POU2F2* in healthy donor $CD19^+$ cells induced transcription of *SPIB* and suppressed transcription of *PRDM1* and *IRF4*. Chromatin immunoprecipitation analysis in BCWM.1 WM cells confirmed binding of POU2F2 and POU2AF1 in *SPIB* and *ID2* promoters. These findings establish a molecular hierarchy among *POU2F2*, *SPIB* and *ID2* during B-cell differentiation, and suggest that aberrant expression of these transcription factors plays an important role in arresting plasmacytic differentiation in WM.

**Keywords:** waldenström Macroglobulinaemia, *POU2F2*, *POU1AF1*, *SPIB*.

Waldenström macroglobulinaemia (WM) is a lymphoplasmacytic lymphoma characterized by accumulation of lymphoplasmacytic cells (LPC) in the bone marrow (BM), and production of an IgM monoclonal protein (Owen *et al*, 2003; Swerdlow *et al*, 2008). Waldenström macroglobulinaemia LPC exhibit deficiencies that impede the differentiation of mature B-cells to plasma cells. The differentiation of normal human B-cells to plasma cells is a carefully orchestrated process, central to which is *PRDM1* (*BLIMP1*). *PRDM1*, through modulation of *BCL6*, *PAX5*, *IRF4* and *XBP1*, regulates B-cell receptor signalling, proliferation control, expression of various B-cell surface proteins and B-lymphoid specific factors, somatic hypermutation and class-switch recombination. The absence of *PRDM1*, or a spliced form of its downstream regulatory gene *XBP1* ($XBP1_{sp}$) results in arrest of B-cell differentiation at the mature B-cell stage and/or lymphoplasmacytic stage of B-cell development (Calame, 2008). The expression of *PRDM1* itself is induced by multiple transcription factors such as the *SP1* and *SP3* isoforms and early growth response 1 (*EGR1*) gene, and repressed by *BCL6*, *PAX5* and the *ETS*-family (v-ets erythroblastosis virus E26 oncogene homolog 1 family) member *SPIB* (Shaffer *et al*, 2000, 2002; Mora-Lopez *et al*, 2008; Schmidlin *et al*, 2008). Reciprocally, transcription of *BCL6*, *SPIB* and *PAX5* is directly repressed by *PRDM1* in mice, thus creating autoregulatory loops between these factors (Shaffer *et al*, 2002). Therefore, aberrant expression of these transcriptional factors could suppress terminal differentiation of LPC and apoptosis. In view of its importance in LPC differentiation, we previously investigated the *PRDM1/XBP1* pathway for transcriptional expression, complemented by gene sequence analysis in WM LPC (Hatjiharissi *et al*, 2007; Leleu *et al*, 2009). We observed considerable heterogeneity in transcription levels of *PRDM1*, *PAX5*, and $XBP1_{sp}$ in sorted BM LPC, though no

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mutations in *PRDM1*, *PAX5* and *XBP1* were identified (Leleu *et al*, 2009). Significant differences in expression levels of several genes were detected in gene expression studies, highlighted by over-expression of the *ETS*-family member *SPIB* (Hatjiharissi *et al*, 2007). Several members of the *ETS*-family have been implicated in B-cell development and differentiation, such as *SPIB*, *SPI1* (*PU.1*) and *ETS1*. However, there are no reports for *ETS* family transcription factors playing a role in the pathogenesis of WM. We therefore investigated the role of *SPIB* and its transcriptional regulators on normal B-cell and LPC differentiation, and report the outcome of these studies.

## Patients and methods

### *Patients*

Lymphoplasmacytic cells were isolated from bone marrow aspirates obtained from 12 previously untreated patients who met the clinicopathological diagnosis of WM using consensus and World Health Organization criteria (Owen *et al*, 2003; Swerdlow *et al*, 2008). All patients provided written consent for use of their samples, and the study was approved by the Dana–Farber Cancer Institute Institutional Review Board. The clinical characteristics for the 12 patients whose LPC were used in these studies are provided in Table I. *MYD88* L265P genotyping in BM WM LPC was performed using allele-specific polymerase chain reaction (PCR) analysis (Xu *et al*, 2013).

### *B-cell isolations and cultures*

$CD19^+$ B-cells from the peripheral blood (PB) of healthy donors and BM $CD19^+$ cells from WM patients were isolated by positive selection using magnetic-activated cell sorting CD19-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). All cultures were performed in Iscove's modified Dulbecco medium (IMDM, Invitrogen, Carlsbad, CA, USA) and 10% fetal calf serum (FCS). Purified B cells were plated at 5 × 105/ml in 96-well culture-plates, and cultured with various combinations of cytokines for 5 d as indicated: interleukin (IL) 2 (20 μ/ml), IL10 (50 ng/ml) and IL15 (10 ng/ml). 10 μg/ml of phosphorothioate CpG oligodeoxynucleotide 2006 (Sigma-Aldrich, St Louis, MO, USA) was also added at the start of culture. To generate plasmablast cells, at day 6 of culture, the cells were harvested, washed and seeded with various combinations of cytokines: IL2 (20 μ/ml), IL6 (50 ng/ml), IL10 (50 ng/ml), IL15 (10 ng/ml), and mouse anti-CD40L IgG (1 μg/ml).

**Table I.** Clinicopathological characteristics of WM patients enrolled in this study.

| | Median | Range |
|---|---|---|
| Age (years) | 66 | 52–79 |
| Gender (Male/Female) | 9/3 | N/A |
| Bone marrow disease (%) | 60 | 10–95 |
| Serum IgM (g/l) | 39·1 | 3·21–64·0 |
| Serum IgA (g/l) | 0·94 | 0·1–3·6 |
| Serum IgG (g/l) | 7·58 | 0·72–25·1 |
| Serum $B_2$-microglobulin (mg/l) | 2·7 | 1·8–12·5 |
| Hematocrit (%) | 31·5 | 22·5–41·7 |
| Extramedullary disease (%) | 4 (33·3%) | N/A |
| *MYD88* L265P | 11 (91·7%) | N/A |

N/A, not applicable.

### *Flow cytometry*

Monoclonal antibodies against human CD19, CD20, CD38, and CD138 directly conjugated with pycoerythrin (PE), PE or PE-cyanin 5 (Cy5) and fluorescein isothiocyanate (FITC), respectively, were purchased from BD Biosciences PharMingen (San Diego, CA, USA). Apoptosis assays were performed by using Annexin V and propidium iodide (PI) [allopycocyanin (APC) annexin V and PI, BD Biosciences PharMingen]. Samples were analysed by flow cytometry on an LSRFortessa (BD Biosciences PharMingen) and analysed using FlowJo software (TreeStar, Ashland, OR, USA).

### *Lentiviral constructs and transductions*

For over-expressing SPIB and POU2F2, lentiviral vector pLenti-SPIB-IRES-eGFP, pLenti-SPIB~ER-IRES-eGFP, and pLenti-POU2F2 -IRES-eGFP were constructed, respectively. pLenti-SPIB~ER-IRES-GFP was constructed by inserting the fusion of the C-terminus of full-length SPIB to a truncated murine oestrogen receptor (ER) before the IRES-eGFP cassette. For knock-down experiments, pLenti-SPIB short hairpin RNA (shRNA)-eGFP vectors, pLenti-POU2F2 shRNA-eGFP, and pLenti-Neg shRNA-eGFP vector encode individual shRNAs directed against *SPIB* (*SPIB* shRNA#1 target sequence 5′-gcattccagctaccctgat-3′, *SPIB* shRNA#2 target sequence 5′-gatcgctgtgtgtctgtaa-3′), *POU2F2* (*POU2F2* shRNA#1 target sequence 5′-gctccagaaataagaatgt-3′, *POU2F2* shRNA#2 target sequence 5′-gcacaacagttactacctt-3′) and a scrambled shRNA, respectively.

Recombinant lentiviral particles were generated by transient co-transfection of HEK-293T cells in a 100-mm plate with 10 μg lentiviral vector, 6·7 μg psPAX2 packaging vector containing *gag-pol*, and envelope vectors through the use of FuGENE®6 transfection reagent according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN, USA). At 48-h post-transfection, the cell supernatant containing the pseudotyped lentiviral particles was collected and concentrated by ultra-centrifugation. The viral vector stocks were titrated on HEK-293T cells for determination of titres. BCWM.1 WM cells or primary WM cells were transduced with the recombinant lentiviral particles at the same multiplicity of infection in the presence of polybrene (10 μg/ml; Sigma-Aldrich). Transduced cells were sorted out as green cells with Fluorescence-activated cell sorting (FACS; BD

FACSAria III cell sorter). To induce translocation of ER fusion proteins, cells were treated with 0·5 μmol/l 4-hydroxytamoxifen (4HT; Sigma-Aldrich).

### *Immunoblot analysis*

Western blotting was performed with antibodies against human SPIB (kindly provided by Lee Ann Sinha, State University of New York, Buffalo, NY, USA or SPIB N-16, Santa Cruz Biotechnology, Santa Cruz, CA, USA), PRDM1 (6D3), POU2F2 (C-20), POU2AF1 (C-20), XBP1(M-186), oestrogen receptor (ER) (MC-20), ETS1(N-276), GAPDH (0411; Santa Cruz Biotechnology), or IRF4, ID2 (D39E8, Cell Signalling Technology, Boston, MA, USA).

### *Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)*

Total mRNA was isolated from cells using RNeasy mini kit (Qiagen, Valencia, CA, USA) and reverse-transcribed into cDNA by using High capacity archive (Applied Biosystems, Foster City, CA, USA). We used a power SYBR green mastermix (Applied Biosystems) for quantitative PCR. Each sample was analysed in duplicate, and expression levels were normalized to *ACTB* expression.

### *Chromatin immunoprecipitation*

Chromatin immunoprecipitation (ChIP) was performed according to an adapted version of the EZ-Magna ChIPTM G kit protocol (MILLIPORE, Temecula, CA, USA). A total of 5 × 107 BCWM.1 cells were used for each immunoprecipitation. Immunoprecipitation was performed with 3 μg polyclonal anti-POU2F2 antibody, or polyclonal anti-POU2AF1(Bob 1) antibody, or 3 μg normal rabbit IgG (Santa Cruz Biotechnology). Precipitated chromatin was purified with QIAmp DNA mini kit (Qiagen) analysed by SYBRgreen real time PCR. Each ChIP was performed in triplicate and each real time PCR reaction in duplicate.

### *Statistical analysis*

Statistical analysis was conducted using Mann Whitney *U* test. Linear correlation and regression analysis was performed with Spearman's rank correlation. Calculations were performed using R (R Foundation for Statistical Computing, Vienna, Austria). A *P*-value ≤0·05 was deemed to be significant.

## Results

### *Aberrant transcriptional and protein expression of SPIB and ID2 in WM*

Given that we have previously observed over-expression of the *ETS*-family members *SPIB*, *ETS1* and *IRF4* using genome expression profiling (GEP) (Hatjiharissi *et al*, 2007), we first validated these findings using quantitative real-time RT-PCR analysis on a separate sample set of WM patients and healthy donors. By comparing $CD19^+$ selected cells from 12 untreated WM patients *versus* age-matched healthy donors, we observed 2·5-fold and threefold higher transcript levels for *SPIB* and *ETS1* in WM patient samples ($P = 0{\cdot}012$ and $P < 0{\cdot}001$, respectively). In contrast, no significant changes in transcript levels for other closely related *ETS*-family members such as *SPI1*, *SPIC*, or *ETV6* were observed (Fig 1A). Transcript levels of *IRF4* and *PRDM1* were decreased by twofold in WM samples ($P = 0{\cdot}008$ and $P = 0{\cdot}0277$ respectively), while levels of $XBP1_{sp}$ were not significantly changed from those of the healthy controls (Fig 1B). *SPIB* has been reported to facilitate plasmacytoid dendritic cell development via E-family factor *TCF4* (*E2-2*) down-regulation of endogenous inhibitors of DNA binding protein 2 (*ID2*), thereby antagonizing E-protein activity (Cisse *et al*, 2008; Nagasawa *et al*, 2008). We therefore investigated whether *ID2*, *TCF4*, and other E-family (*ELS-PBP1*, *TCF3 and TCF12*) as well as the ID-family members (*ID1*, *ID3 and ID4*) were aberrantly expressed in WM. By real-time PCR, only *ID2* and *ID1* transcript levels showed a significant change (decrease >threefold, $P < 0{\cdot}001$ for both *ID2* and *ID1*) in WM *versus* normal donor cells (Fig 1C). The increased expression of SPIB, and decreased expression of ID2 was also observed in WM cells by Western blot analysis (Fig 1D).

### *Over-expression of* SPIB *inhibits healthy donor peripheral blood B-cells differentiation into plasma cells*

To clarify functions of *SPIB* and *ID2* in normal B-cell differentiation, we next subjected healthy donor peripheral blood $CD19^+$ B-cells to *in vitro* induced plasma cell differentiation by 7-d cytokine incubation, after which $CD38^+$ CD20- and CD38-CD20- cells were sorted out. We observed significant down-regulation of *SPIB*, along with up-regulation of *ID2* and *PRDM1* in $CD38^+$ CD20- plasmablastic cells ($P < 0{\cdot}001$ for all studies, Fig 2A).

To further investigate the role of *SPIB* during B-cell differentiation, *SPIB* was constitutively expressed in transduced primary B-cells derived from healthy donor peripheral blood. Three days post-transduction with a lentiviral vector harbouring the *SPIB* coding sequence upstream of the IRES-GFP cassette, $CD19^+$ B cells were cultured under differentiation conditions. Results showed that *in vitro* induced plasma cell differentiation following cytokine incubation was significantly repressed in *SPIB* transduced B-cells ($P < 0{\cdot}001$; Fig 2B). Importantly, *SPIB* over-expression was associated with down-regulation of $XBP1_{sp}$ and *IRF4* ($P < 0{\cdot}001$; Fig 2C). Furthermore, we transduced primary B-cells using a lentiviral vector so as to simultaneously knock-down *ID2* and over-express *SPIB*. Compared to cells overexpressing *SPIB* alone, the simultaneous knock-down of *ID2* and over-expression of

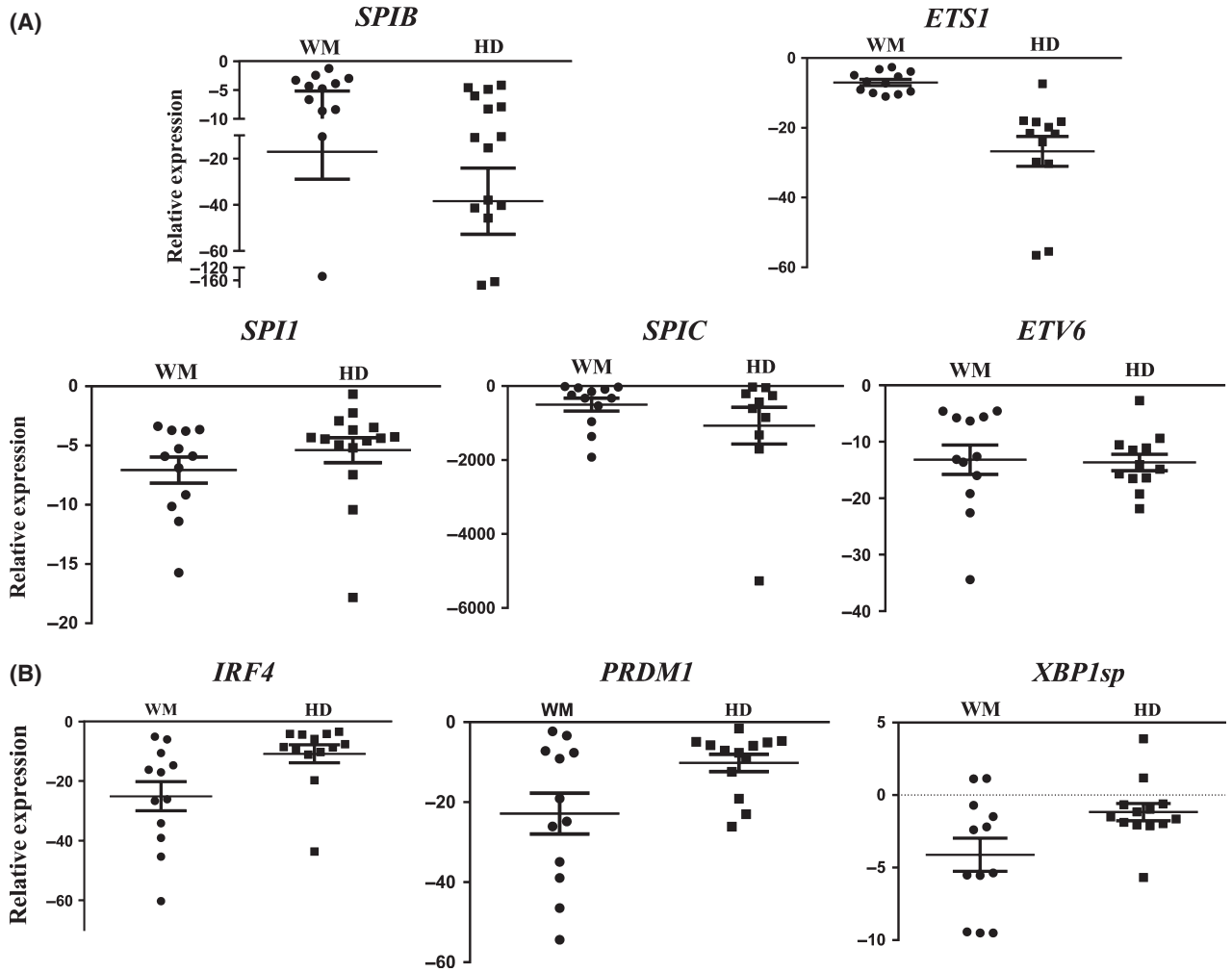


**Fig 1.** Aberrant expression of *SPIB*, *ID2, POU2F2,* and *POU2AF1* in WM. Relative expression of *SPIB*, *SPI1*, *ETS1*, *ETV6* (A); *IRF4*, *PRDM1*, $XBP1_{sp}$ (B); *ID1* and *ID2* (C) transcripts in bone marrow (BM) $CD19^{+}$ cells from 12 untreated Waldenström macroglobulinaemia patients (WM) and 15 healthy donors (HD) using quantitative real-time PCR analysis. Transcripts levels are shown relative to *ACTB* expression. $P = 0{\cdot}012$ (*SPIB*); $P < 0{\cdot}001$ (*ETS1*); $P = 0{\cdot}008$ (*IRF4*) and $P = 0{\cdot}0277$ (*PRDM1*) for comparisons of WM to healthy donor cells, but otherwise is non-significant. Western blot findings (D) showing differences in transcription factor protein expression in BM lymphoplasmacytic cells from WM patients (WM1–4) and healthy donors (HD1, HD2).

*SPIB* did not reveal any differences in *PRDM1*, $XBP1_{sp}$, or *IRF4* expression (data not shown).

### *Knock-down of endogenous* SPIB *in primary WM cells facilitates plasma cell differentiation*

The requirement of down-regulation of *SPIB* in normal B-cell differentiation implicates that *SPIB* over-expression in WM cells may also block plasma cell differentiation by enforcing suppression of *PRDM1*, $XBP1_{sp}$ and *IRF4*. To elucidate the impact of *SPIB* on the regulation of the plasma cell gene expression programme in WM, we first transduced BCWM.1 WM cells with a control vector, a vector containing *SPIB-EREG* fusion, and a vector containing *SPIB* shRNA (Fig 3A). All viral constructs harboured the GFP reporter for the convenience of sorting out green cells after transduction. Compared with cells transduced with the control virus, expression levels of *SPIB* were increased 16-fold and decreased 2·5-fold following overexpression or knock-down of *SPIB* respectively ($P < 0{\cdot}05$; Fig 3B). *PRDM1*, $XBP1_{sp}$ and *IRF4* levels were decreased in cells overexpressing *SPIB*, whereas only *IRF4* was significantly increased in cells with *SPIB* knocked down. Interestingly, *ID2* levels were also increased following the knock-down of *SPIB*, but no change was observed with over-expression of *SPIB* (Fig 3B). We next knocked down *SPIB* in primary BM LPC cells taken from untreated WM patients, and used real-time RT-PCR analysis to confirm decreased *SPIB* expression levels in transduced primary WM LPC. Knock-down of *SPIB* led to a 2·5-fold increase in plasma cell differentiation ($P < 0{\cdot}05$; Fig 4A). These findings were accompanied with significantly increased expression of *PRDM1*, *IRF4* and $XBP1_{sp}$ ($P < 0{\cdot}05$;

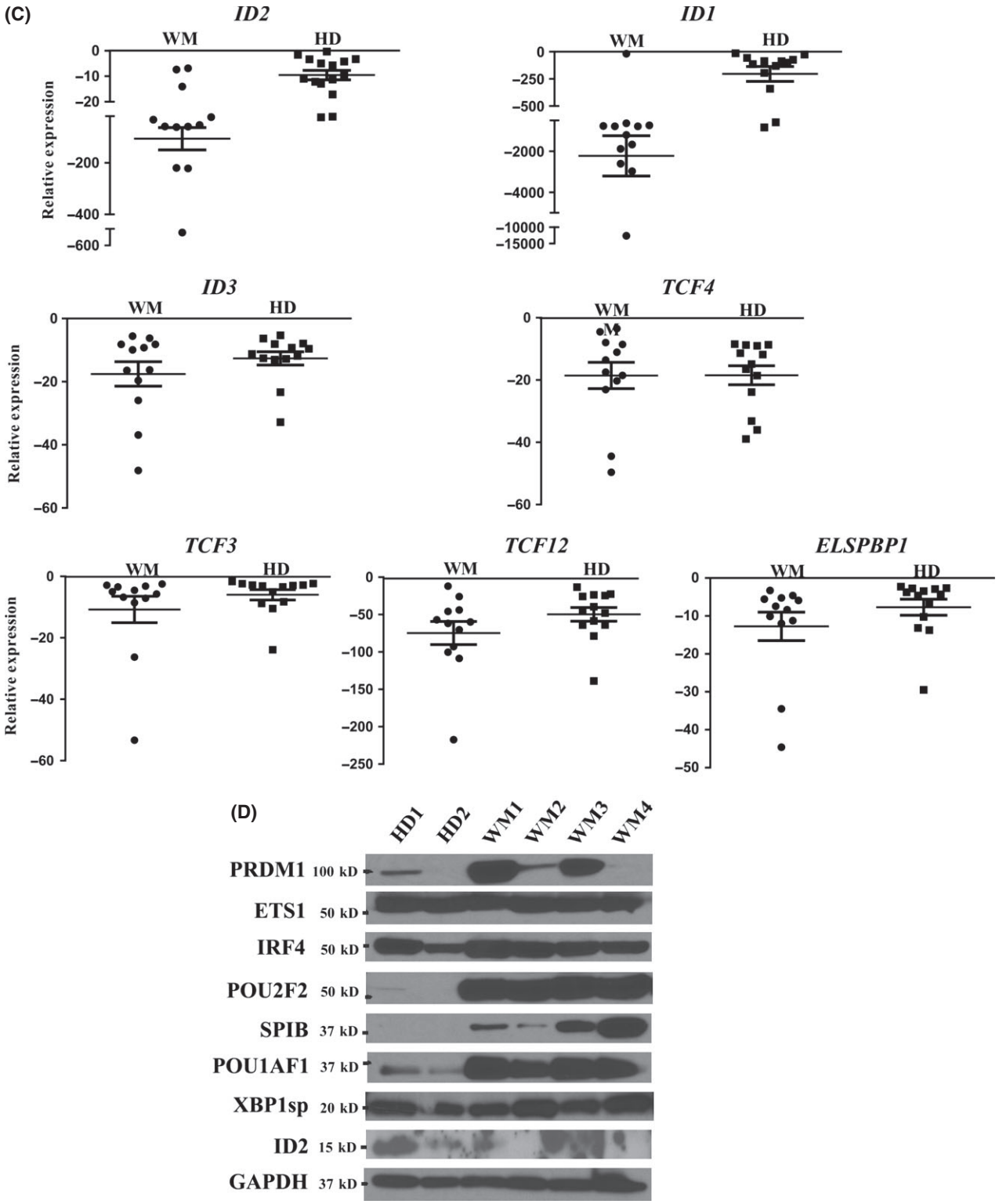


**Fig 1.** (Continued).

Fig 4B). Consistent with the results obtained by the knock-down of *SPIB* in BCWM.1 WM cells, *ID2* levels were significantly increased following *SPIB* knock-down. Importantly, the level of the anti-apoptotic transcription factor, *BCL2* was also decreased after *SPIB* was knocked down ($P < 0{\cdot}05$; Fig 4B).

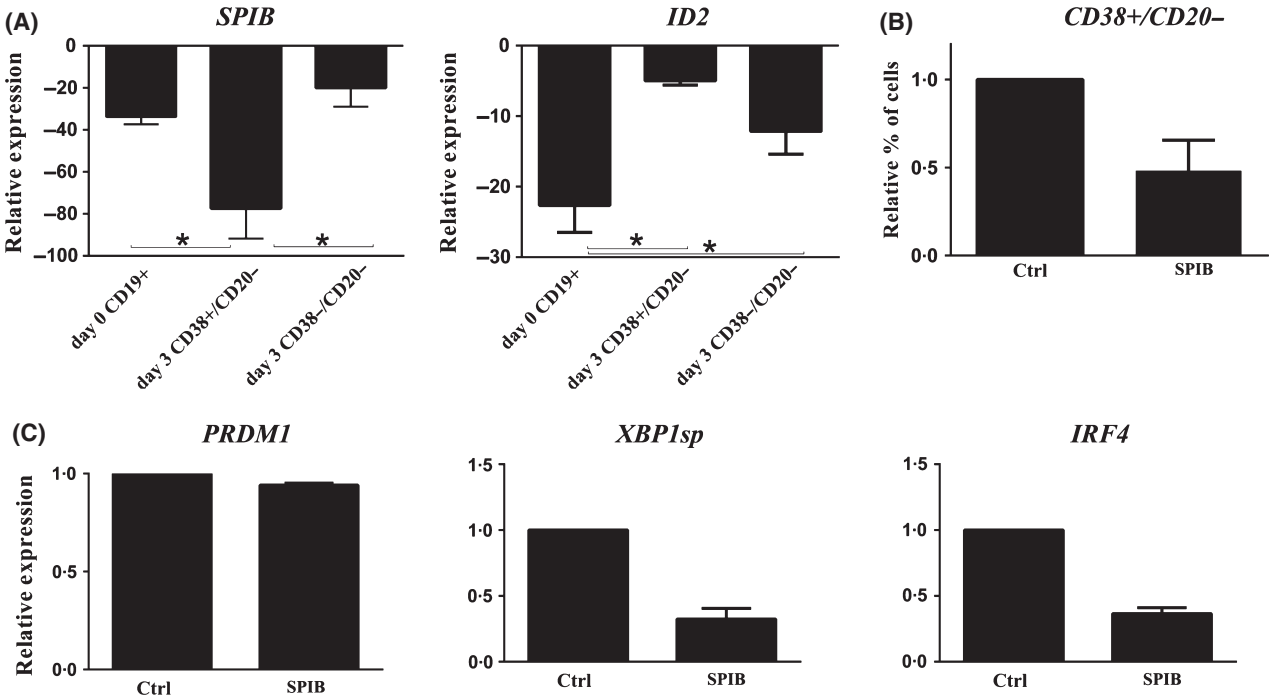


**Fig 2.** Inhibition of primary human CD19$^+$ cell differentiation into plasma cells by ectopic expression of *SPIB*. Relative expression of *SPIB* and *ID2* following cytokine induced plasma cell (CD38$^+$/CD20-) differentiation of primary CD19$^+$ B-cells from healthy donor peripheral blood (A) (*$P < 0{\cdot}001$). Transcriptional expression shown is relative to *ACTB* levels and results of five independent experiments are shown. After CD19$^+$ cells from peripheral blood were transduced with recombinant lentiviral vectors and then induced to differentiate for 4 d, GFP+ cells were analysed for CD38 and CD20 surface expression by flow cytometry (B). Percentages of CD38$^+$/CD20- cells in SPIB transduced cells (SPIB) were normalized to cells transduced with the control lentiviral vector (Ctrl). Mean ± standard deviation values of six independent experiments are shown ($P < 0{\cdot}001$). Repression of *IRF4* and $XBP1_{sp}$ transcription by *SPIB* (C). Representative results of three experiments are shown ($P < 0{\cdot}001$).

### *Aberrant expression of* POU2F2 *is related to the abnormal expression of* SPIB *and* ID2 *in WM*

By whole genome sequencing of LPC from 30 WM patients, no somatic mutations, copy number alterations or translocations affecting *SPIB* were identified (Hunter *et al*, 2014). These findings suggested to us that *SPIB* over-expression was probably a secondary oncogenic event in WM LPC. Little is known about the dynamic regulation of *SPIB* during human B-cell differentiation. It has been reported that in mice *SPIB* is a direct target of the co-activator *POU2AF1*, and the conserved octamer site (5′-ATGCAAAT-3′) in the *SPIB* downstream promoter (P2) is essential for allowing the binding of a ternary complex containing *POU2AF1* together with POU proteins POU2F2 or POU2F1 (Bartholdy *et al*, 2006). Strikingly, we found threefold higher mRNA levels of *POU2F2* in WM LPC compared to healthy donor BM B-cells in our previous GEP studies (Hatjiharissi *et al*, 2007) and in the real time RT-PCR validation analysis reported here in ($P = 0{\cdot}0011$; Fig 5A). Western blot analysis demonstrated increased protein expression of POU2F2, as well as POU2AF1 despite no differences in transcription, suggesting post-transcriptional regulatory changes, in WM LPC *versus* healthy donor B-cells (Fig 1D). Interestingly, by real time RT-PCR, we also observed that elevated levels of *POU2F2* associated with increased levels of *SPIB*, and lower levels of *ID2* in WM patients (Fig 5B). Higher levels of *POU2F2* and *SPIB* transcription were also associated with increased bone marrow disease burden and serum IgM levels, as well as decreased haematocrit ($P < 0{\cdot}01$). No other significant correlations for *POU2F2* and *SPIB* with other baseline patient characteristics depicted in Table I were identified.

Consistent with the result that *SPIB* is a direct target of *POU2AF1* in mice (Lenz *et al*, 2008), we next showed that *POU2F2* and *POU2AF1* are bound to *SPIB* promoter 2 octamer sites in BCWM.1 WM cells by chromatin immunoprecipitation (ChIP) analysis. Furthermore, we showed that *POU2AF1* and *POU2F2* bound to the dual octamer sites in the elusive promoter region of *ID2*, but not the elusive promoter of *ID1* and *ID3* (Fig 5C). Give these findings, we next knocked down *POU2F2* in BCWM.1 WM cells with lentiviral transduction. Compared with cells transduced with the control virus, decreased expression of *SPIB*, *ID2*, *ID1* and *BCL2* resulted following *POU2F2* knock-down in BCWM.1 cells (Fig 5D). On the other hand, ectopic expression of *POU2F2* in primary peripheral blood CD19$^+$ cells from healthy donors showed increased expression of *SPIB*, along with decreased levels of *PRDM1* and *IRF4* (Fig 5E). Taken together, the above data suggest that there is a molecular

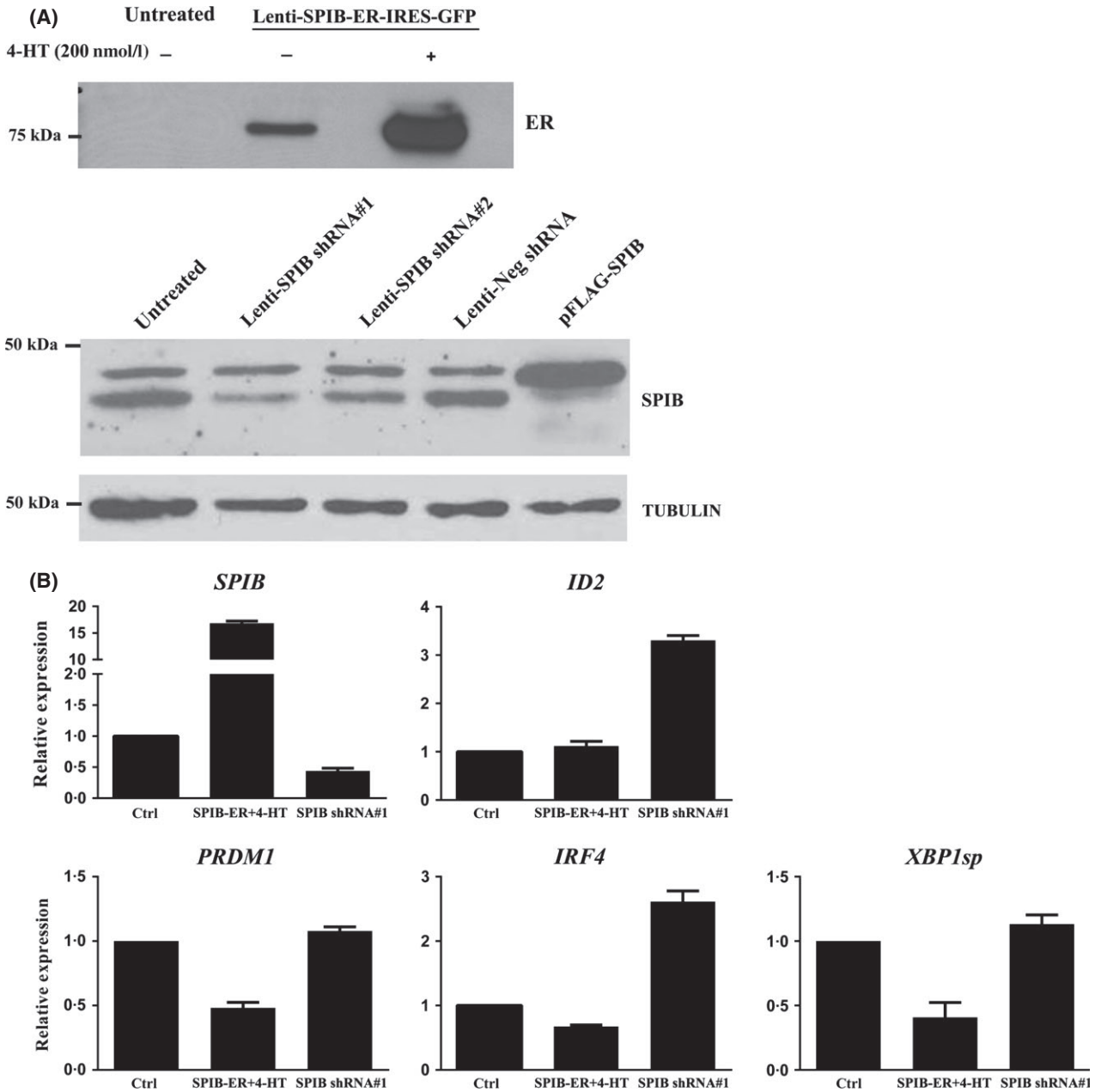


**Fig 3.** Relative gene expressions in the BCWM.1 WM cells over-expressing *SPIB* or knocking down endogenous *SPIB*. Transcriptional activity following over-expression and knocking-down of *SPIB* in BCWM.1 Waldenström macroglobulinaemia (WM) cells (A). *PRDM1*, $XBP1_{sp}$, and *IRF4* levels were decreased in cells overexpressing *SPIB*, whereas *IRF4* and *ID2* levels were significantly increased in cells with *SPIB* knocked down (B). GFP+ cells in transduced WM cells were sorted out and relative mRNA levels of genes were analysed by using quantitative real-time PCR. Representative results of three experiments are shown ($P < 0{\cdot}05$).

hierarchy among *POU2F2*, *SPIB*, and *ID2*, and that abnormal expression of *POU2F2* along with *SPIB* and *ID2* leads to repression of plasmacytic differentiation in WM.

## Discussion

Here in, we report the aberrant transcriptional and protein expression of *POU2F2*, *SPIB*, and *ID2* in WM LPC by comparison to healthy donor CD19-selected cells. It remains possible that these observations reflect comparisons brought on by differences in the stage of B-cell differentiation for healthy donor cells *versus* WM LPC. The use of CD19-selected B-cells has been traditionally used for such comparative studies. However, as recently shown by us, differences in micro-RNA profiling exist based on whether $CD19^+$ or $CD19^+$ $CD27^+$ selection is used in comparative studies of healthy donor B-cells *versus* WM LPC (Cao *et al*, 2011). Whether $CD19^+$ $CD27^+$ (memory B-cells) represent the

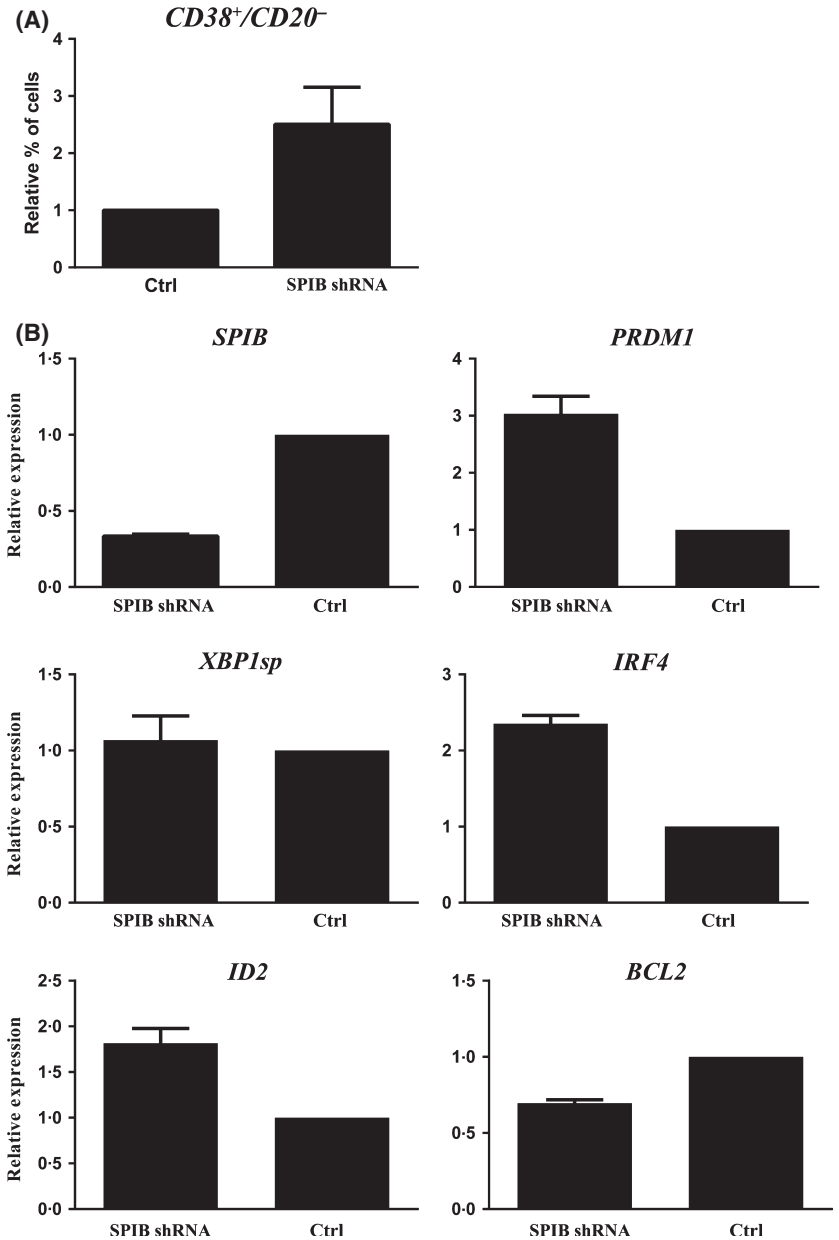


**Fig 4.** Induction differentiation of transduced primary WM cells by knocking-down *SPIB*. Knock-down of *SPIB* in primary Waldenström macroglobulinaemia (WM) lymphoplasmacytic cells induces $CD38^+$ CD20- plasmacytic cell formation (A). Waldenström macroglobulinaemia bone marrow $CD19^+$ cells isolated from untreated WM patients were transduced with control lentiviral particles (Ctrl) or the vector for knocking-down *SPIB* and cultured under the differentiation condition. Mean ± standard deviation (SD) values of three independent experiments are shown ($P < 0{\cdot}05$). Knock-down of *SPIB* induced $CD38^+$ CD20- plasmablast formation and relationship to expression of *PRDM1*, *XBP1sp*, *IRF4*, and *ID2* is shown (B). Mean ± SD values of three independent experiments is shown ($P < 0{\cdot}05$).

appropriate normal phenotypic analogue of WM remains to be determined.

Knock-down of *SPIB* in primary WM cells promoted plasma cell differentiation, which accompanied increased expression levels of *PRDM1*, *IRF4* and *XBP1sp*, and decreased level of *ID2*. Importantly, the level of the anti-apoptotic transcription factor, *BCL2* was also decreased after *SPIB* was knocked down. Conversely, using healthy donor peripheral blood B-cells, we showed that *SPIB* over-expression blocked plasma cell differentiation, which was associated with down-regulation of *PRDM1*, $XBP1_{sp}$ and *IRF4*. The aberrant expression of *SPIB* has been implicated as an oncogenic event in activated B-cell (ABC) subtype of Diffuse Large B-cell Lymphoma (DLBCL). Higher levels of *SPIB* have been observed in ABC DLBCL *versus* germinal centre (GCB) B-cell DLBCL, which share a gene expression profile similar to WM/LPL cells (Staudt & Dave, 2005; Lenz *et al*, 2007). Knock-down of *SPIB* decreases proliferation and survival in ABC but not GCB DLBCL cell lines (Lenz *et al*, 2007). The oncogenic basis for over-expression of *SPIB* is related to amplification in many cases of ABC DLBCL, though translocations effecting *SPIB* expression have also been reported (Lenz *et al*, 2007, 2008). In OCI-Ly3 ABC DLBCL cells, high *SPIB* transcription levels occur as a result of a translocation of the intact *SPIB* coding region to an Ig3′α enhancer (Lenz *et al*, 2007). *MYD88* L265P mutations are found in both WM and ABC subtype DLBCL tumours, and it may be possible that this mutation contributes to the dysregulation of *SPIB* either

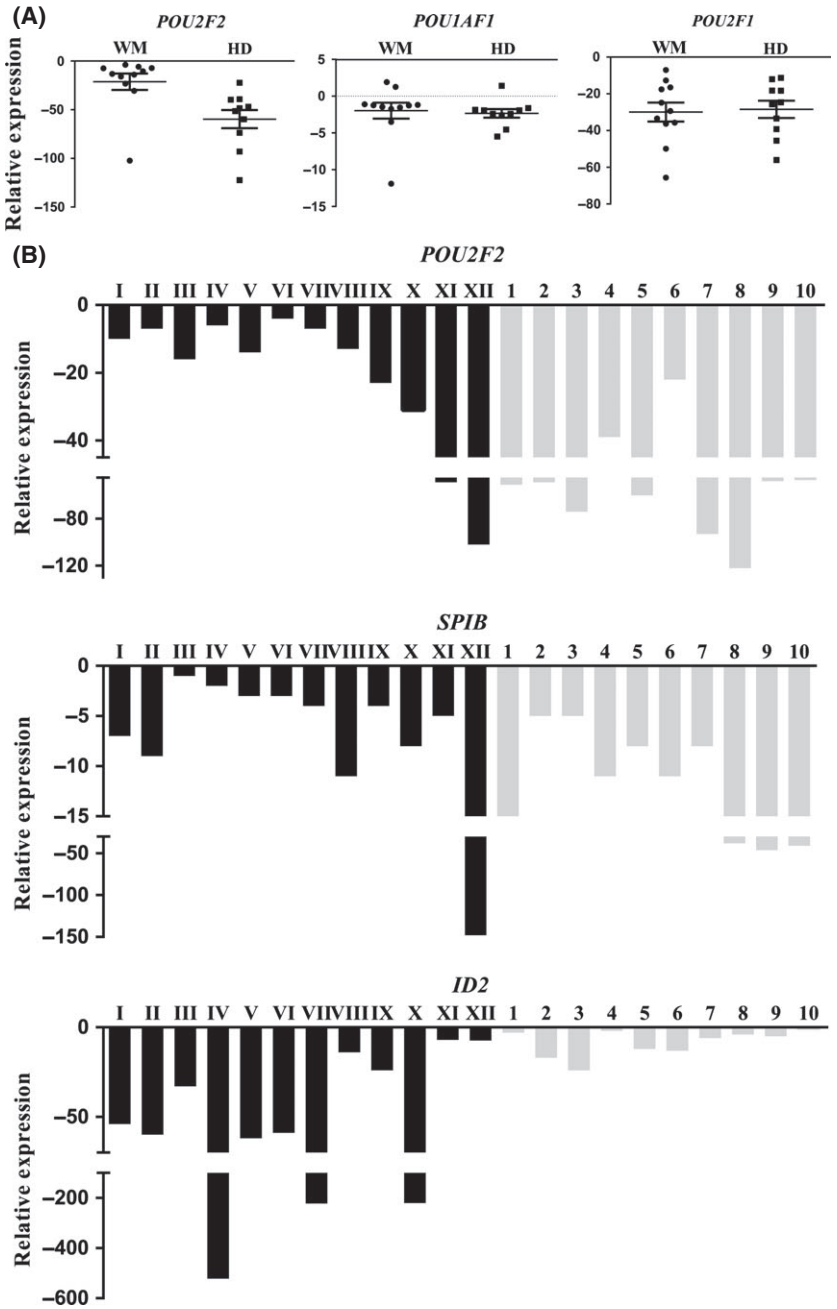


**Fig 5.** Aberrant expression of *POU2F2* in WM is related abnormal expressions of *SPIB/ID2* and is responsible for repressing plasmacytic differentiation of WM cells. Aberrant expression of *POU2F2* transcript, but not *POU2AF1* and *POU2F1* (A), associates with increased and decreased levels of SPIB and ID2 respectively in bone marrow $CD19^+$ cells from 12 untreated Waldenström macroglobulinaemia (WM) patients (I–XII) compared to 10 healthy donors (HD; 1–10) (B). POU2F2 and POU2AF1 bind to the promoter of *SPIB* and *ID2* with ChIP analysis in BCWM.1 WM cells (C, D). Mean ± standard deviation (SD)values of three independent experiments are shown ($P < 0{\cdot}05$). Binding sites of primers for CHIP analysis and putative octamer sites for *POU2F2* and *POU2AF1* binding are shown in the schematic representation of the 5′ region of the human *ID1*, *ID2*, *ID3*, and *SPIB* locus. Mutagenesis of the dual octamer sites (-ATGTAAAT GTAGTTTCAAT ATGCAAAT-) in the elusive *POU2F2* promoter region released the repression of transcription activity in the dual luciferase assay. Knocking down *POU2F2* in BCWM.1 cells caused decreased expression of *SPIB* and *BCL2* (E). Representative results of three experiments are shown. Over-expression of *POU2F2* in primary $CD19^+$ B-cells increases the expression of *SPIB* and decreases the expression of *IRF4* and *PRDM1* (F). Mean ± SD values of three independent experiments are shown ($P < 0{\cdot}05$).

directly or indirectly via *POU2F2* as the findings of our study suggest (Ngo *et al*, 2011; Treon *et al*, 2012). No somatic mutations, copy number alterations or translocations impacting *POU2F2* or *SPIB* were identified in WM patients using whole genome sequencing (Hunter *et al*, 2014). Further studies to clarify the relationship and contribution of *MYD88* L265P as well as other identified WM somatic variants, including *CXCR4* (*WHIM*) mutations, to *POU2F2* and *SPIB* dys-regulation are needed (Hunter *et al*, 2014). In contrast to ABC DLBCL, *IRF4* expression in WM cells was reduced. *IRF4* over-expression has been implicated as a countermeasure to the toxic β-interferon production induced by *MYD88* L265P that is induced by aberrant B-cell receptor signalling, mediated by mutations in *CARD11* and *CD79A* that are common in ABC DLBCL, but rare or absent in WM (Yang *et al*, 2012). Both ABC DLBCL and WM patients show responses to lenalidomide, which suppresses *SPIB* expression through interactions with cereblon, highlighting the potential to develop targeted therapies against *SPIB* in diseases, such as ABC DLBCL and WM, that depend on this regulatory factor for growth and survival (Treon *et al*, 2008; Hernandez-IIizatriurri *et al*, 2011; Yang *et al*, 2012).

As part of these studies, we also investigated the potential contribution of other *ETS*-family members to WM

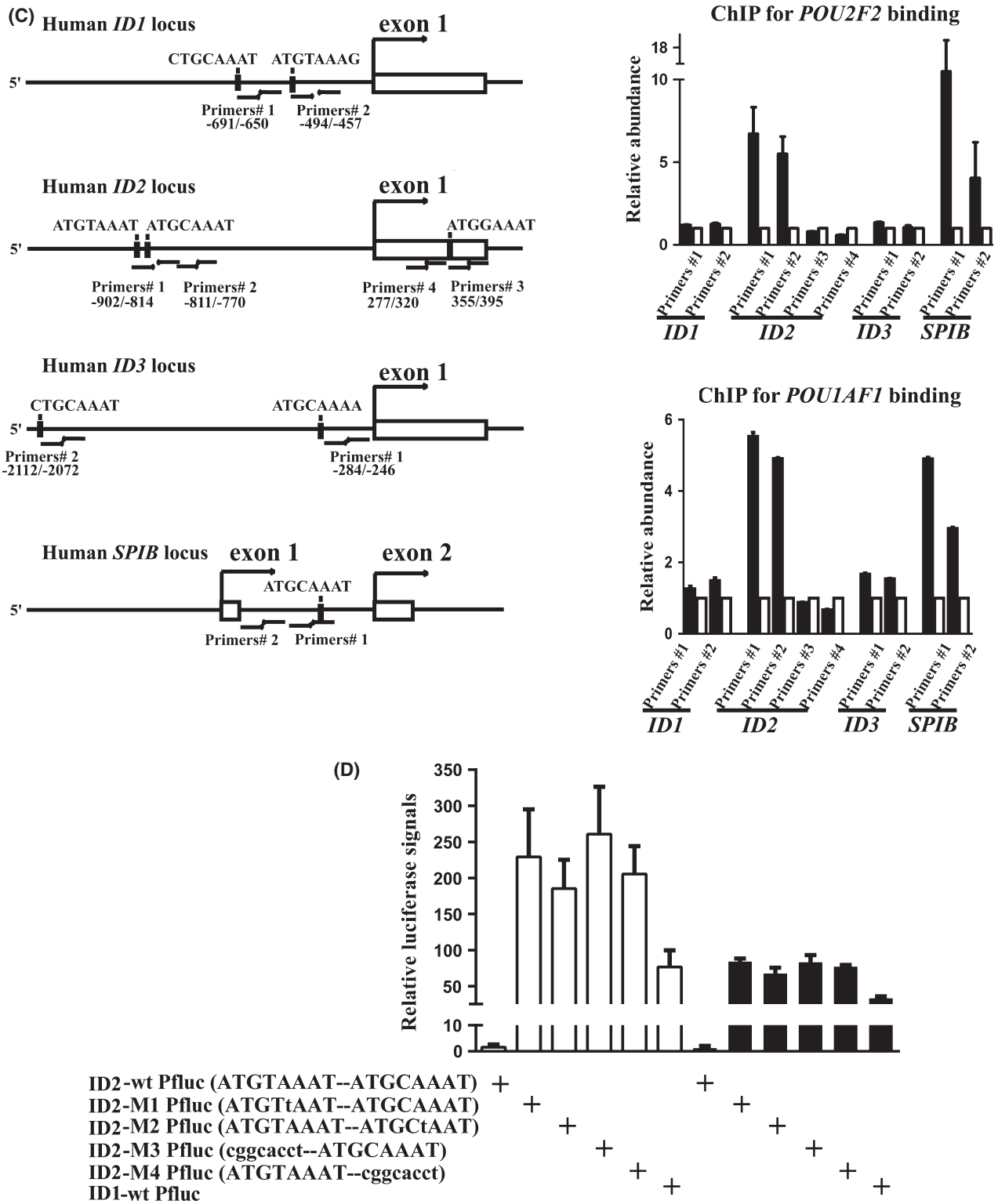


Fig 5. (Continued).

pathogenesis. We did not observe any significant difference in *SPI1* levels *versus* normal B-cells. While *SPIB* and *SPI1* are highly related, they are known to play non-redundant roles in B cell development and differentiation by activation or repression of different target genes. Compared with *SPI1*, as well as other *ETS* factors, *SPIB* has a unique proline/serine/threonine-rich region (amino acid 31–61) in the N-terminus which may confer to unique target gene and regulatory

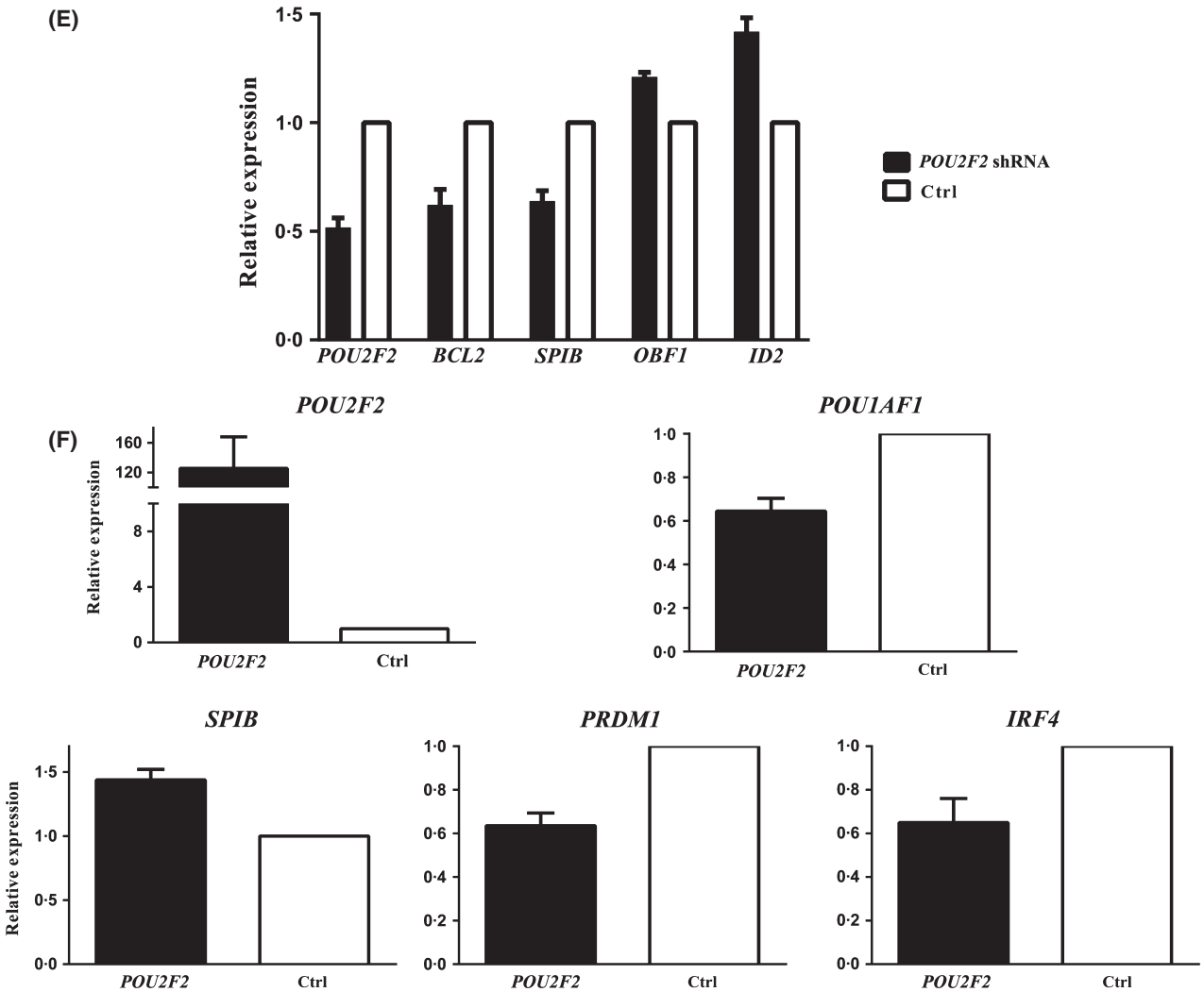


Fig 5. (Continued).

cofactor binding (Rao *et al*, 1999). Future experiments designed to clarify the functions imparted by the different domains of *SPIB* will hopefully provide insights into the regulation and functions of *SPIB* to WM pathogenesis, and potentially permit the design of specific therapeutic molecules.

Another *ETS* factor, *ETS1* has also been implicated in murine B-cell differentiation by directly interacting with *PRDM1* and blocking its DNA binding activity (John *et al*, 2008). Based upon the observation that *ETS1* and *SPIB* are differentially expressed in naïve and GC B cells, it has been proposed that *SPIB* and *ETS1* might prevent premature differentiation of activated GC and naïve B cells respectively (Schmidlin *et al*, 2008). In the context of WM, the finding of increased protein level of SPIB but not ETS1 suggests that *SPIB* may play a more important role in the pathogenesis of WM.

Recently, it has been reported that the network of *SPIB*, *ID2* and *TCF4* are crucial for plasmacytoid dendritic cell (pDC) development (Cisse *et al*, 2008; Nagasawa *et al*, 2008). It has been suggested that *SPIB* may contribute to the pDC development by promoting *TCF4* activity, which results in the suppression of *ID2* expression (Cisse *et al*, 2008). Moreover, *TCF4* controls *SPIB* expression by binding to a conserved site in the *SPIB* first intron (Cisse *et al*, 2008). In addition to the aberrant expression of *SPIB* and *ID2* in WM, we also observed that *ID2* levels were significantly increased in $CD38^{+}$/CD20- plasmacytic cells, which coincided with decreased *SPIB* levels. Confirming this relationship, *ID2* expression levels were increased following knock-down of *SPIB* in our studies. Our studies also confirmed that POU2F2 and POU2AF1 directly bound to the promoter of *ID2*. These findings therefore provide a molecular link among *POU2F2*, *SPIB* and *ID2*. It is possible that SPIB may contribute to *ID2* expression by interacting with the complex of POU2F2 and POU2AF1. Alternatively, SPIB may complex with other proteins such as *TCF4* or other E proteins to regulate the expression of *ID2*.

It has been reported that, in mice, *SPIB* is a direct target of the co-activator *POU2AF1*. The conserved octamer site (5′-ATGCAAAT-3′) in the *SPIB* downstream promoter (P2) is essential for the direct binding of the POU2AF1/POU2F2 or POU2F1 complex (Cisse *et al*, 2008). *POU2AF1* itself is a direct target of *XBP1* and decreased $XBP1_{sp}$ resulted in decreased expression of *POU2AF1* during plasma cell differentiation in mouse cells (Shen & Hendershot, 2007). Strikingly, we found increased protein expression of both POU2F2 and POU2AF1 in WM than in normal controls. In the case of POU2AF1, post-transcriptional regulation is likely to play a role for these findings because transcriptional activity showed no differences *versus* healthy donor B-cells. By functioning synergistically with *POU2AF1*, *POU2F2* is able to maintain the gene expression profile of a mouse Ig-secreting plasmacytoma in plasmacytoma x T lymphoma hybrids. This implicates that both factors may be essential to the specialized functions of the Ig-secreting plasmacytes (Salas & Eckhardt, 2003). In this study, we confirmed that *POU2F2,* together with POU2AF1 binds to the promoter of *SPIB* in WM cells. Over-expression and knockdown of *POU2F2* increased and decreased the expression of *SPIB*, respectively. These findings help provide an important molecular link among *POU2F2*, *SPIB* and plasma cell differentiation.

In conclusion, the findings of this study show that *POU2F2*, *SPIB* and *ID2* are aberrantly expressed in LPC from patients with WM, and regulate B-cell differentiation to plasma cells. The results confirm a role for *POU2F2*, *SPIB*, and *ID2* in the blockade of terminal differentiation in WM.

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## Author contributions

YZ, XL, and SPT conceived and designed the experiment, and wrote the manuscript. YZ, SPT, ZRH and RJM performed the data analysis. LX, YC, BTC, and CJP prepared samples. YZ, XL, GY, and LX performed validation studies. SPT provided patient care, obtained consent and samples.

## Conflict of interest disclosures

The authors have no conflicts of interest to report.

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