1 **The BLIMP1 – EZH2 nexus in a non-Hodgkin lymphoma**

2 **Running title: BLIMP1 and EZH2 in lymphoma**

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24

25 **Abstract**

26

27 Waldenström's macroglobulinemia (WM) is a non-Hodgkin lymphoma, resulting in
 28 antibody-secreting lymphoplasmacytic cells in the bone marrow and pathologies
 29 resulting from high levels of monoclonal immunoglobulin M (IgM) in the blood.
 30 Despite the key role for BLIMP1 in plasma cell maturation and antibody secretion, its
 31 potential role in WM cell biology has not yet been explored. Here we provide
 32 evidence of a crucial role for BLIMP1 in the survival of WM cells and further
 33 demonstrate that BLIMP1 is necessary for the expression of the histone
 34 methyltransferase EZH2 in both WM and multiple myeloma. The effect of BLIMP1
 35 on EZH2 levels is post translational, at least partially through the regulation of
 36 proteasomal targeting of EZH2. Chromatin immunoprecipitation analysis and
 37 transcriptome profiling suggest that the two factors co-operate in regulating genes
 38 involved in cancer cell immune evasion. Co-cultures of natural killer cells and WM
 39 cells further reveal that both factors participate directly in immune evasion, promoting
 40 escape from natural killer cell mediated cytotoxicity. Together, the interplay of
 41 BLIMP1 and EZH2 plays a vital role in promoting the survival of WM cells.

42

43 **Introduction**

44

45 Waldenström's macroglobulinemia (WM) is a rare plasma cell dyscrasia with around
 46 3-6 people per million diagnosed annually world wide [1-4]. It is characterised by the
 47 expansion of a monoclonal population of malignant cells in the bone marrow with a
 48 lymphoplasmacytic character, that is, cellular phenotypes ranging from that of B-
 49 lymphocytes to overt plasma cells that exhibit hypersecretion of immunoglobulin M

50 (IgM) [5]. A large proportion of WM symptoms arise because of high levels of IgM
51 paraprotein in patients' blood and tissues [6]. Curiously, over 90% of WM tumours
52 carry an activating mutation in the signaling adaptor MYD88, typically L265P that
53 serves as a key oncogenic driver in the disease [7-9].

54

55 The transcription factor B-lymphocyte induced maturation protein-1
56 (BLIMP1) drives plasma cell differentiation, mediating transcriptional changes via
57 the recruitment of co-factors to chromatin [10-12]. During plasma cell maturation,
58 BLIMP1 represses key B-lymphocyte identity factors and signalling mediators [13,
59 14], while simultaneously driving plasma cell specific gene expression and antibody
60 secretion [15-17]. Depending on the mouse model system used, BLIMP1 appears to
61 be required for the survival of long-lived plasma cells in the bone marrow and
62 promotes multiple myeloma (MM) cell survival [16, 18-20]. Conversely, BLIMP1
63 functions as a tumour suppressor in diffuse large B cell lymphoma (DLBCL),
64 consistent with its repression of proliferation genes during plasma cell differentiation
65 [15, 21-23]. Consistent with a potential tumour suppressor role, WM tumours harbour
66 frequent heterozygous losses of *PRDM1*, the gene encoding BLIMP1 [24]. However,
67 BLIMP1 is expressed in a subset of WM lymphoplasmacytic cells [25, 26], in line
68 with its necessity for antibody secretion [17, 19, 27], a critical aspect of WM
69 pathology. Furthermore, BLIMP1 is induced downstream of toll like receptor
70 engagement and MYD88 [28, 29] and *PRDM1* mRNA is elevated in tumours
71 harbouring the MYD88^{L265P} mutation [30], which is associated with poorer prognosis
72 in WM [31].

73

Also important in plasma cell differentiation, enhancer of zeste 2 (EZH2) is both a physical and genetic interaction partner of BLIMP1 [17, 32]. The interaction was first suggested in mouse primordial germ cells, where BLIMP1 and EZH2 share a highly overlapping set of binding sites [33, 34]. EZH2 is the catalytic component of the polycomb repressive complex 2, placing methyl groups on lysine 27 of histone 3, typically tri-methylation (H3K27me3), to repress transcription [35, 36]. While EZH2 is essential for embryonic development [37], it has frequent activating mutations in DLBCL, and is commonly overexpressed in MM, making it a promising therapeutic target [38-40]. Surprisingly, while aberrant regulation of histone modifications has been implicated in WM pathogenesis [41], the role of EZH2 is yet to be investigated.

In this study, we examined the role of BLIMP1 in WM, and its potential interplay with EZH2. We demonstrate for the first time that BLIMP1 regulates WM cell survival and maintains EZH2 protein levels. We identify a large overlap in transcriptional targets of the two factors and show that they repress transcription on an overlapping set of genes in a parallel fashion. In a highly novel finding, we reveal roles for BLIMP1 and EZH2 in evasion from natural-killer (NK) cell mediated cytotoxicity, with BLIMP1 suppressing NK cell activation in response to WM cells and both factors suppressing NK cell mediated WM cell death. Thus, inhibition of BLIMP1 or EZH2 could be a promising therapeutic strategy. Together, our data highlights the multifaceted roles of the BLIMP1-EZH2 nexus in WM cell survival.

Materials and Methods

RNA isolation, cDNA synthesis and RT-qPCR

99

100 Cells were lysed in TRIsure reagent (#BIO-38032, Bioline, London, UK), and RNA
101 was extracted according to the manufacturer's instructions. cDNA synthesis and RT-
102 qPCR are described in supplementary materials and methods.

103

104 **Viability and reduction assays**

105

106 The percentage of live cells was determined by trypan blue exclusion assay (Thermo
107 Fisher Scientific, USA). The per cent reduction was determined by resazurin assay
108 (#sc-206037, Santa Cruz Biotechnology, CA, USA).

109

110 **Proteasomal inhibition**

111

112 To inhibit proteasome activity, the cells were treated for 4 h with 5 μ M MG-132 (#sc-
113 201270, Santa Cruz Biotechnology, dissolved in DMSO), 20 h post dox addition.

114

115 **RNAseq and ChIPseq**

116

117 Prior to RNAseq, The *PmiR2* and *NTmiR* RP cells were treated with 0.2 μ g/mL dox
118 for 48 h. RP cells were treated with 300nM tazemetostat (EPZ-6438, #S7128,
119 Selleckchem, Munich, Germany) or DMSO for 48 h. Transcription factor ChIP for
120 BLIMP1 and EZH2 was performed as previously described [33, 42, 43]. Histone
121 ChIP for pan-H3 and the H3K27me3 mark was performed as previously described
122 [44].

123

124 **NK cell isolation, degranulation and cytotoxicity assays**

125

126 NK cells were isolated from heparinised buffy coats obtained from healthy human
127 donors, who all provided informed consent, provided by the Icelandic Blood Bank
128 (ethical approval #06-068).

129

130 Additional details for Western blotting, immunofluorescence, reduction assays,
131 RNAseq, ChIPseq, NK cell isolation, degranulation and cytotoxicity assays are
132 described in Supplementary Materials and Methods.

133

134 **Results**

135

136 **BLIMP1 is important for cell survival in Waldenström's macroglobulinemia**

137 As BLIMP1 is expressed in a subset of WM patients' lymphoplasmacytic cells [25,
138 26], and given its crucial roles in antibody secretion and plasma cell differentiation,
139 we wanted to determine whether it plays a role in WM cell biology. We compared
140 BLIMP1 expression in the myeloma cell line OPM-2 [45] to that of three WM cell
141 lines RPCI-WM1 (RP), MWCL-1 (MW) and BCWM.1 (BC) by immunofluorescence
142 staining (Fig. 1A). All of the RP cells expressed BLIMP1, while the MW and BC
143 cells had more heterogeneous expression, with 43% and 18% of the cells expressing
144 high levels of BLIMP1 respectively (Fig. 1A right panel). This heterogeneity of
145 BLIMP1 expression is interesting because the antibody-secreting cells responsible for
146 the IgM associated pathology of WM are likely dependent on BLIMP1 for secretion.
147 We subsequently chose to focus mainly on the RP cell line as it had the most uniform
148 BLIMP1 expression.

149

150 To knock-down (KD) BLIMP1, we engineered RP cells with two distinct
 151 doxycycline (dox)-inducible artificial miRNAs targeting *PRDM1* mRNA (*PmiR1* and
 152 *PmiR2*), or a non-targeting control miRNA (*NTmiR*), and MW cells with *NTmiR* and
 153 *PmiR1*. The induction of *PmiR1* and *PmiR2* led to the loss of BLIMP1 protein in RP
 154 cells (Fig. 1B) and *PmiR1* led to a 60% reduction in MW cells (Fig. 1C). The KD
 155 resulted in decreased cell survival, with 58% and 72% live cells remaining relative to
 156 *NTmiR* in RP *PmiR1* and *PmiR2* cell cultures respectively, 48 h post dox addition
 157 (Fig. 1D). No viable cells remained 6 days after induction of *PmiR1* or *PmiR2* (Fig.
 158 S1A). MW *PmiR1* cells also displayed an initial decrease in viability to 71% of the
 159 *NTmiR* (Fig. 1D), but by day 5 had recovered their viability, perhaps due to the
 160 survival of the non-BLIMP1 expressing cellular compartment. The proportion of
 161 apoptotic cells increased in RP cells by 2.6- and 2.3-fold 48h post *PmiR1* and *PmiR2*
 162 induction respectively (Fig. S1B). Although we consistently saw an increase in
 163 Annexin-V positive MW cells upon BLIMP1-KD, it was not statistically significant
 164 (Fig. S1C).

165

166 Crucially, the decreased cell viability upon BLIMP1-KD in RP cells was rescued
 167 in *PmiR1* cells transduced with miR-resistant BLIMP1 from a lentiviral construct [46]
 168 (Fig. 1E). BLIMP1 overexpression resulted in 81% viability, relative to BLIMP1-
 169 transduced *NTmiR* control, whereas EGFP-transduced cells were only 65% viable at
 170 48 h post *PmiR1* induction (Fig. 1F). Five days post BLIMP1 KD, 57% of the
 171 BLIMP1-transduced cells were viable, compared to only 3% of the EGFP-transduced
 172 cells (Fig. 1F), further reflected in an increased reduction capacity of the BLIMP1

173 complemented cells (23% vs. 6% resazurin reduction) (Fig. 1G). Taken together,
174 BLIMP1 is a survival factor in WM cells, likely due to the suppression of apoptosis.

175

176 **BLIMP1 expression maintains EZH2 protein levels**

177 As studies of mouse plasmablasts and germ cells have shown a functional overlap
178 and direct interaction of BLIMP1 and EZH2 [17, 33, 34], we investigated the
179 potential interplay between the two factors in WM and MM. Our first line of inquiry
180 revealed a decrease in EZH2 protein expression upon BLIMP1 KD in RP cells (Fig.
181 2A) and in OPM-2 MM cells also engineered with inducible *PmiR1* (Fig. 2B).
182 Genetic complementation of miR expression with miR-resistant BLIMP1 (Fig. 1E)
183 restored EZH2 protein levels in RP cells, confirming the effect is specifically due to
184 BLIMP1 depletion (Fig. 2C, quantified in Fig. S2A). Furthermore, BLIMP1 and
185 EZH2 levels were positively correlated in two separate complementation experiments
186 ($R^2 = 0.338$, $R^2 = 0.684$) based on quantitation of nuclear fluorescent signal. Taken
187 together these data reveal for the first time, a dependency of EZH2 expression on
188 BLIMP1.

189

190 Reverse transcription followed by quantitative PCR (RT-qPCR) revealed that
191 *EZH2* mRNA levels were unchanged in RP *PmiR2* cells upon BLIMP1 KD and only
192 slightly decreased in OPM-2 cells upon *PmiR1* induction, indicating that the loss of
193 EZH2 expression is at the post-transcriptional level (Fig. 2D-E). Notably, the
194 treatment of RP *PmiR1* cells with the proteasome inhibitor MG-132, restored EZH2 to
195 the same level as that of *NTmiR* cells also treated with proteasome inhibitor (Fig. 2F-
196 G), revealing that BLIMP1 modulates EZH2 by inhibition of proteasome mediated
197 degradation.

198

199 However, complementing the RP *PmiR1* cells with ectopic EZH2 expression (Fig.
200 2H) failed to increase their viability (Fig. 2I). Consistent with this, the EZH2-specific
201 catalytic inhibitor tazemetostat did not affect RP cell viability even over a 96 h period
202 (Fig. S2B), despite a dose dependent decrease in H3K27me3 levels (Fig. 2J), nor did
203 7 days of treatment. Taken together, BLIMP1 maintains EZH2 protein levels via
204 modulation of proteasome mediated degradation, but the effect of BLIMP1 on cell
205 survival is independent of EZH2.

206

207 **BLIMP1 KD induces large transcriptional changes**

208 To investigate the overlap of BLIMP1 and EZH2 in downstream gene regulation in
209 WM cells, we performed transcriptome profiling of the RP *PmiR2* and *NTmiR* cells
210 following 48 h of induction. Using a q-value cutoff of 0.05, we identified 7814
211 differentially expressed genes between *PmiR2* and *NTmiR* (Fig. 3A and Table S1).

212 Consistent with BLIMP1 repressing the B cell transcriptional program [15, 17, 19,
213 47], previously characterised B cell lineage targets of BLIMP1, including *CIITA* [48,
214 49], *PAX5* [50], *SPIB* and *BCL6* [15] were repressed by BLIMP1 in our study (Fig.
215 3B). However, other BLIMP1 targets such as *MYC* [51] and *ID3* [15] were unaltered.
216 Curiously, the myeloma-driving transcription factor *IRF4* [44], which is activated
217 downstream of BLIMP1 in plasma cells [17] was repressed by BLIMP1 in RP cells
218 (Fig. 3B). Interestingly, the BTK inhibitor, *IBTK* was also repressed by BLIMP1. The
219 inhibition of BTK via Ibrutinib is now a common treatment for WM [52]. The
220 expression of key apoptosis genes was increased following BLIMP1 KD (Fig. 3C)
221 and finally, we also observed a de-repression of *SMURF2* mRNA (Fig. S3A).
222 *SMURF2* is an E3 ubiquitin ligase known to target EZH2 for proteasome-mediated

223 degradation and could therefore contribute to the decrease in EZH2 protein levels
224 upon the loss of BLIMP1 [53]. Taken together, BLIMP1 KD induces extensive gene
225 expression changes in RP cells including the de-repression of B cell- and apoptosis-
226 related genes, as well as *SMURF2*.

227

228 **BLIMP1 and EZH2 regulate overlapping pathways**

229 Because of the dependency of EZH2 on BLIMP1 levels, we wanted to determine
230 to which extent the effect of BLIMP1 on transcription is dependent on EZH2's
231 catalytic activity, and therefore performed transcriptome profiling upon catalytic
232 inhibition of EZH2 with tazemetostat. This resulted in 450 differentially expressed
233 genes compared to vehicle alone (Fig. 3D and Table S2). The amplitude of change for
234 individual genes was smaller than the changes induced by BLIMP1 KD (Comparing
235 Fig. 3D and 3A). Nevertheless, a highly significant overlap emerged between
236 transcripts increased in the BLIMP1 KD and tazemetostat treated cells (184 genes, p
237 = $1.8e-37$) (Fig. 3E, Table S3), but the genes with decreased expression did not
238 significantly overlap (Fig. 3F, Table S4). Tazemetostat led to the de-repression of B
239 cell identity genes, including *STAT5B* and *ID3* (Fig. S3B) and interestingly, despite
240 EZH2 inhibition not affecting cell survival, a number of apoptosis genes were
241 differentially expressed, including *XAF1*, *CASP4*, *FAS* and *JUN* (Fig. S3C),
242 suggesting a possible sensitisation to apoptosis.

243

244 To investigate the pathways jointly regulated by BLIMP1 and EZH2, we
245 performed an overlap analysis using the molecular signatures database [54, 55] of the
246 184 genes induced by both BLIMP1 KD and tazemetostat. Several gene sets were
247 significantly enriched, including interferon and TNF α responses, the inflammatory

248 response and apoptosis (Fig. 3G), consistent with the known roles of BLIMP1 [14, 18,
249 56-58], and suggesting that pathways involved in immune responses are regulated in
250 concert by BLIMP1 and EZH2. Collectively, the transcriptomic analyses demonstrate
251 a large overlap in targets of repression by BLIMP1 and EZH2, highlighting the
252 interplay of the two factors.

253

254 **BLIMP1 binds to a set of H3K27me3 marked genes at a distance from the** 255 **mark**

256 Given the overlap in genome wide positioning of BLIMP1 and the H3K27me3
257 mark in mouse plasmablasts [17], we examined whether the overlapping effects of
258 BLIMP1 and EZH2 on gene transcription could be modulated not only through the
259 regulation of EZH2 by BLIMP1 but also by BLIMP1 potentially recruiting EZH2 to
260 chromatin in WM cells. We therefore performed chromatin immunoprecipitation
261 coupled to deep sequencing (ChIPseq) for H3K27me3 and BLIMP1 in RP cells. We
262 identified 14946 H3K27me3 peaks (Table S5), assigned to 4198 genes (Table S6),
263 and 506 BLIMP1 peaks (Table S7), assigned to 841 genes (Table S8). If BLIMP1
264 recruits EZH2 to chromatin in WM cells, a large proportion of BLIMP1 peaks would
265 be located in close proximity to H3K27me3 marks. However, only 8 sites bore both
266 H3K27me3 and BLIMP1 within 500 bp (Fig. 4A) and only a small level of bimodal
267 enrichment of H2K27me3 was observed in the ± 3 kb flanking BLIMP1 peaks (Fig.
268 4B). Interestingly however, when we compared the genes assigned to the peaks in the
269 respective experiments, 261 genes were both bound BLIMP1 and marked by
270 H3K27me3 in RP cells, a highly statistically significant overlap ($p = 2.9e-25$) (Fig.
271 4C, Table S9). An analysis [54, 55] of the genes marked by both BLIMP1 and
272 H3K27me3 in the RP cells demonstrated significant enrichment for the same immune

273 signalling gene sets as in the RNAseq data, as well as genes relating to the estrogen
274 response, hypoxia and the p53 pathway (Fig. S4A).

275

276 To test whether these findings apply also to MM, we performed ChIPseq for
277 BLIMP1 and H3K27me3 in the OPM-2 (Tables S10-13) and NCI-H929 (Tables S14-
278 17) myeloma cell lines, and EZH2 in NCI-H929 cells (Tables S18-19). As with the
279 RP cells, we rarely observed peaks within a 500bp distance when comparing BLIMP1
280 and H3K27me3 (Fig. S4B) or BLIMP1 and EZH2 (Fig. S4C) in the OPM-2 and NCI-
281 H929 cell lines. However, we observed a statistically significant overlap between
282 genes bound by BLIMP1 and H3K27me3 in NCI-H929 cells, but not OPM-2 cells
283 (Fig. S4D). Genes bound both by BLIMP1 and EZH2 were not statistically over-
284 represented in NCI-H929 cells (Fig. S4E). The above analyses reveal that the majority
285 of BLIMP1 and EZH2 binding to chromatin occurs at relatively distant sites both in
286 MM and WM cells. Thus, their direct regulation on chromatin is unlikely to be due to
287 a direct physical interaction.

288

289 The known DNA binding motif for BLIMP1 [59] was enriched by *de novo* motif
290 analysis in the BLIMP1 peaks for all three cell lines (Fig. S4F) and the BLIMP1
291 ChIPseq signals were enriched at proximal positions relative to their assigned
292 transcription start sites (TSSs) (Fig. S4G). Comparing the distribution of BLIMP1
293 peaks in OPM-2 and NCI-H929 to the RP cell line (Fig. S4H), showed that BLIMP1
294 binds to largely the same sites in WM and MM.

295

296 An analysis [54, 55] of genes assigned to H3K27me3 peaks for each cell line
297 revealed similar gene sets marked by H3K27me3 in all three cell lines (Figure S5A).

298 Interestingly, TNF α and IL2/STAT5 signalling genes were amongst the most highly
299 enriched in the RP but not the myeloma cells. The myeloma cell lines displayed a
300 much stronger enrichment of H3K27me3 over TSSs than the RP cells (Fig. S5B),
301 indicating that H3K27me3 is more often present at gene distal sites in RP cells than
302 myeloma cells. EZH2 was also highly enriched just downstream of TSSs in NCI-
303 H929 cells (Fig. S5C). Correspondingly, we observed low enrichment of the
304 H3K27me3 mark in the OPM-2 and NCI-H929 cell lines over sites marked by
305 H3K27me3 in the RP cell line (Fig. S5D). Interestingly, *SMURF2* which can affect
306 EZH2 stability [53], was bound by BLIMP1 in all three cell lines and bore the
307 H3K27me3 mark in the RP cells as well as EZH2 in the NCI-H929 cell line (Fig.
308 S5E).

309

310 To identify the direct transcriptional targets of BLIMP1 and EZH2, we compared
311 our ChIPseq and transcriptome profiling data from the RP cell line. There were 231
312 and 120 genes associated with BLIMP1 binding that were either induced or repressed
313 upon BLIMP1 KD respectively (Fig. 4F-G). Conversely, 118 genes were both
314 induced upon tazemetostat treatment and associated with the H3K27me3 mark (Fig.
315 4H), but only 7 genes with decreased expression were marked by H3K27me3 (Fig.
316 4I). Overall, only 3% of genes marked by H3K27me3 were de-repressed following
317 tazemetostat treatment in these experiments, indicating that inhibition of EZH2's
318 catalytic activity alone is insufficient to activate most H3K27me3 targets over 48 h.
319 Meanwhile, approximately one third of BLIMP1-bound genes showed altered
320 expression upon BLIMP1 KD, indicating that BLIMP1 binding actively maintains
321 gene repression. Taken together, as BLIMP1 and H3K27me3 are largely present at
322 distinct sites from one another, and most of their overlapping effects on transcription

are likely through binding to the same genes at distinct sites, as well as BLIMP1 maintenance of EZH2 protein levels.

A subset of BLIMP1 targets are regulated via EZH2

A number of genes repressed by BLIMP1 bore both BLIMP1 and the H3K27me3 mark, such as *PIK3CD*, *POU2F2* (Fig. 4D) and *ZFP36L1* (Fig. 5A). Meanwhile, others bore only the H3K27me3 mark, but not BLIMP1, including *RCAN3*, *TNFRSF14* and *CIITA* (Fig. 5B-D). Yet others such as the macrophage gene *TFEC* [60] were bound by BLIMP1 alone (Fig. 5E). We therefore asked whether the genes bearing the H3K27me3 mark are repressed by BLIMP1 via the maintenance of EZH2. By overexpressing EZH2 in RP cells upon BLIMP1 KD, we performed RT-qPCR and observed that repression of the genes *RCAN3* and *ZFP36L1* was restored (Fig. 5F) as well as the repression of the immune inhibitory checkpoint ligand/receptor gene *TNFRSF14* [61] and the inhibitory receptor gene *HAVCR2* [62]. By comparison, BLIMP1 binding targets not bearing the H3K27me3 mark such as *TFEC* were not altered upon EZH2 restoration. Thus, a subset of BLIMP1 targets are regulated through EZH2 maintenance and can be identified by the H3K27me3 mark, whereas others are regulated independently of EZH2.

BLIMP1 represses transcription of immune surveillance and signalling molecules in concert with EZH2

Further analysis of the highly enriched immune signalling genes differentially expressed upon BLIMP1 KD identified three mechanistic categories. First, BLIMP1 represses the expression of genes encoding surface ligands that can activate T and NK cells, including *ICOSLG*, *TNFSF9*, *CD48*, *MICA*, *CLEC2B*, *ICAM1* and *ITGAM*, and

348 MHC class II molecules including *HLA-DMA*, *HLA-DMB* and *CD1D* (Fig. 6A).
 349 Second, BLIMP1 represses genes encoding both immune-checkpoint inhibitory
 350 ligands and their corresponding receptors, including the receptor-ligand pairs
 351 *TNFRSF14* and *BTLA*, as well as *LAG3* and *LGALS3*. Furthermore, the inhibitory
 352 receptor gene *LILRB1*, whose loss promotes immune escape in myeloma [63] is
 353 repressed by BLIMP1. Additionally, the gene encoding the inhibitory ligand PD-L2 is
 354 repressed, with BLIMP1 binding just downstream of *PDCDLG2* (Fig. S6A). Notably,
 355 repression of inhibitory ligands and receptors could promote or inhibit escape from
 356 immune surveillance. The third mechanistic category includes interferon and TNF α
 357 signalling genes. The receptor-encoding genes *IFNGR1*, *IFNAR1*, *IFNLR1*,
 358 *TNFRSF1A* and *TNFRSF1B* are de-repressed upon BLIMP1 KD, as well as
 359 downstream players in interferon signalling, *JAK1*, *STAT1*, *STAT2*, *IRF9* *IFIT1-3*,
 360 *OAS1-3*, and TNF pathway members *MAP3K5*, *CASP8* and *CASP9*, which are also
 361 apoptosis mediators (Fig. 3C). Beyond these categories, MHC class I genes, *HLA-A*
 362 and *HLA-B* which can both mediate inhibition of NK-cell immune surveillance, are
 363 induced by BLIMP1, in contrast to previous studies [58, 64].

364

365 Around half of the genes discussed above were also significantly changed with
 366 tazemetostat treatment (Fig. S6B), including *CD48*, *LILRB1*, *LGALS3*, *PDCD1LG2*
 367 and *STAT1*. Interestingly, while none of the surface molecule genes except for
 368 *PDCDLG2* were bound by BLIMP1, some were enriched for H3K27me3 (Fig. 6B).
 369 Whereas the downstream signalling effectors, *IFIT2* and *STAT1*, were bound by
 370 BLIMP1 (Fig. 6C), consistent with previous findings [65]. In addition, a number of
 371 these differentially expressed genes did not bear either H3K27me3 or BLIMP1, and
 372 their expression changes are therefore likely secondary effects downstream of EZH2

373 and BLIMP1. Taken together, BLIMP1 and EZH2 repress the transcription of genes
374 that encode mediators of killing by NK or T cells when expressed on target cells, NK-
375 and T-cell inhibitory receptors and their ligands, as well as cytokine receptors and
376 downstream signal propagation.

377

378 **BLIMP1 and EZH2 confer evasion from NK cell-mediated cytotoxicity**

379 The transcriptional changes above suggested that BLIMP1 and EZH2 were potentially
380 mediating escape from tumour immune surveillance [66]. We therefore hypothesised
381 that the alterations in gene expression upon the loss of BLIMP1 protein or EZH2
382 activity could lead to changes in NK cell-mediated cytotoxicity. We isolated NK cells
383 from human blood and co-cultured them with RP cells after BLIMP1 KD or treatment
384 with tazemetostat and measured the surface expression of the degranulation marker
385 LAMP-1 (also called CD107a) on NK cells by flow cytometry. This revealed a 1.5-
386 fold increase in frequency of LAMP-1⁺ NK cells upon co-culture with *PmiR1*
387 compared to *NTmiR* RP cells, showing that the loss of BLIMP1 sensitises NK-cells
388 to activation by RP (Fig. 6D-E and S5D), however tazemetostat-treated RP cells did
389 not increase NK-cell degranulation. Strikingly though, both BLIMP1 KD and
390 tazemetostat treatment resulted in increased NK cell-mediated death of RP cells (Fig.
391 6F). Collectively, these data indicate that BLIMP1 and EZH2 drive the escape from
392 NK-cell immune surveillance in WM, with BLIMP1 both suppressing NK cell
393 activation and the resultant WM cell death, whereas EZH2 suppresses the cell death
394 response of WM cells.

395

396 **Discussion**

397

398 In this study we show for the first time that the plasma cell master regulator
399 BLIMP1 has a crucial role in WM cell survival and maintains the protein levels of the
400 histone methyltransferase EZH2, demonstrating a more complex functional
401 interaction between BLIMP1 and EZH2 than previously thought [17, 32-34].
402 Furthermore, we show that the factors mediate the evasion of immune surveillance, as
403 evidenced by the enhanced degranulation of NK cells upon loss of BLIMP1 in WM
404 cells, and enhanced NK cell-mediated WM cellular cytotoxicity upon either BLIMP1
405 KD or EZH2 inhibition.

406

407 Our finding that BLIMP1 promotes survival of WM cells provides new insight
408 into WM biology and potentially its aetiology. BLIMP1 is activated downstream of
409 MYD88 signalling in B cells [29], and BLIMP1 expression is increased in tumours
410 harbouring the MYD88^{L265P} activating mutation in WM [30]. However, heterozygous
411 deletions in the 6q locus containing *PRDM1* are increased in MYD88^{L265P} tumours
412 [24]. As high levels of BLIMP1 inhibit proliferation, and intermediate levels promote
413 survival and immunoglobulin (Ig) secretion [47, 67], our findings might imply that
414 the loss of one copy of the *PRDM1* gene subsequent to MYD88 constitutive
415 activation dampens the anti-proliferative effect of BLIMP1 while still maintaining its
416 positive effect on survival and Ig secretion. Indeed, we observed decreased cell
417 viability upon the loss of BLIMP1 even in MW cells that expresses low BLIMP1
418 levels. A complete loss of BLIMP1 during tumorigenesis has been shown to give rise
419 to B cell lymphomas in mice [21-23], but the loss of Ig secretion ability and plasma
420 cell differentiation would likely preclude WM tumour formation, with the subsequent
421 requirement for BLIMP1 in survival.

422

423 A first line of treatment in WM is rituximab therapy, targeting the B cell
424 specific surface molecule CD20 at least in part through NK-cell engagement [68].
425 However, the plasma cells remaining after this treatment present one of the biggest
426 challenges in WM therapy [69]. Furthermore, rituximab is not recommended for
427 patients exhibiting high serum IgM levels [70], due to the risks of an IgM ‘flare’
428 following treatment [71]. This highlights the importance of the antibody-secreting
429 CD20^{low} or negative plasma cell compartment in WM [69, 72], which is likely
430 maintained by BLIMP1.

431

432 Interestingly, the requirement for BLIMP1 in mediating WM cell survival is
433 through other mechanisms than its effect on EZH2. While both MM and normal
434 plasma cells rely on BLIMP1 for their survival [18, 19, 73], EZH2 expression is
435 decreased in mature plasma cells [74, 75]. Nevertheless, many MM cell lines are
436 reliant on EZH2 for cytokine independent growth and thus their more aggressive
437 plasma cell leukemia phenotype [40, 75]. Our results indicate that the effects of
438 BLIMP1 on EZH2 levels might extend to myeloma, and perhaps other tumours where
439 the two factors are co-expressed, and warrants further study.

440

441 Immune evasion is most extensively studied in relation to solid tumours, but in
442 lymphomas, the paradigm is somewhat different. Lymphocytes interact with other
443 cells of the immune system not only when they are abnormal, such as in cancer, but
444 also to receive stimulatory and inhibitory signals, regulating their own functions [76].
445 In WM, secreted PD-1 ligands inhibit T cell responses [77], but other mechanisms of
446 immune evasion have not been investigated. Our results demonstrate for the first time
447 that BLIMP1 and EZH2 mediate evasion from NK cell surveillance, both in terms of

the suppression of NK cell activation downstream of BLIMP1 as well as resistance to NK cell mediated cytotoxicity downstream of both factors. Based on our transcriptomic profiling, BLIMP1 likely promotes immune evasion in WM by repressing activating ligands and MHC class I molecules leading to “hiding” from cytotoxic lymphocytes. Conversely, EZH2 is more likely to be de-sensitising WM cells to external cytotoxicity-inducing signals perhaps by repressing pro-apoptotic gene expression. Interestingly, in melanoma EZH2 promotes evasion from IFN γ -producing cytotoxic T cells [78]. This finding together with our current study make EZH2 an interesting target for chemical sensitisation of WM and other tumours to immune-cell mediated killing.

In conclusion, we provide evidence for a crucial role of BLIMP1 in promoting WM cell survival and we show that BLIMP1 maintains EZH2 protein levels in both WM and MM cells. We further reveal a cooperation between BLIMP1 and EZH2 in the repression of immune surveillance genes, and find that BLIMP1 and EZH2 confer evasion from NK-cell mediated cytotoxicity. In future studies it would be important to further investigate the intricate interplay between BLIMP1 and EZH2 in cancers to expand their therapeutic potential.

Data availability

RNAseq and ChIPseq data are available at <http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-7739> under the accession code: E-MTAB-7739.

Acknowledgements

We thank Arnar Pálsson and Dagný A. Runarsdóttir for their advice on RNAseq data analysis, Jóna Freysdóttir and Sunnefa Yeatman Omarsdóttir for their advice on the isolation of NK cells, and the group of Eiríkur Steingrímsson for useful discussions and advice on the project. We thank Dr. Roopsha Sengupta for providing critical inputs and proof-reading the manuscript.

Funding

This work was supported by project grants from the Icelandic Centre for Research (RANNIS) (grant no. 140950-051) and the Icelandic Cancer Society, a doctoral fellowship from the University of Iceland, and grant from the University of Iceland Eggertssjódur and funds from the COST Project EpiChemBio.

Competing interests

The authors declare that they have no conflicts of interest.

Figure Legends

Figure 1: BLIMP1 promotes the survival of WM cells

(A) BLIMP1 expression in the myeloma cell line OPM-2 and in WM cell lines RP, MW and BC as detected by immunofluorescence staining, with a bar graph representing percentage of BLIMP1^{bright} cells. Scale bars represent 20µm. (B) Representative immunoblot of BLIMP1 expression following 48h induction of RP cells expressing *NTmiR*, *PmiR1* or *PmiR2*. (C) Immunofluorescence staining of BLIMP1 following 48h induction in MW cells expressing *NTmiR* or *PmiR1*, with

497 CellProfiler quantification. **(D)** Percentage of live cells as determined by Trypan blue
 498 exclusion assay in the RP and MW cell lines comparing *PmiR1* or *PmiR2* to *NTmiR*
 499 following 48h of induction. **(E)** Immunoblot depicting lentiviral ectopic expression of
 500 EGFP or BLIMP1-EGFP in the RP *PmiR1* cells, next to the RP *NTmiR* cells. **(F)** The
 501 percentage of live RP *PmiR1* cells with ectopic EGFP or BLIMP1-EGFP expression
 502 determined by the Trypan blue exclusion assay normalised to the percentage of live
 503 cells following transduction of RP *NTmiR* cells with EGFP or BLIMP1-EGFP.
 504 BLIMP1-EGFP compared to EGFP at Day 2. **(G)** Per cent reduction as measured by
 505 resazurin assay for RP *PmiR1* cells with EGFP compared to BLIMP1-EGFP, five
 506 days after miR induction. All p-values as determined by student's two-tailed t-test. * p
 507 ≤ 0.05 ; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. All graphs were plotted as the
 508 mean of three independent experiments, with error bars representing standard
 509 deviation.

510

511 **Figure 2:** BLIMP1 maintains EZH2 protein levels in WM cells

512 **(A)** Immunoblot of BLIMP1 and EZH2 expression following 48h induction of RP
 513 cells expressing *NTmiR*, *PmiR1* or *PmiR2*. **(B)** Immunoblot of BLIMP1 and EZH2
 514 expression following 48h induction of OPM-2 cells expressing *NTmiR* compared to
 515 *PmiR1*. **(C)** Immunofluorescence staining of BLIMP1 and EZH2 expression
 516 following 48 h induction of RP cells expressing *NTmiR* or *PmiR1*, transduced with
 517 EGFP or BLIMP1-EGFP. Scale bars represent 20 μ m. **(D)** RT-qPCR results depicting
 518 relative mRNA expression of *PRDM1* and *EZH2* normalised to *PPIA* and *ACTB* in
 519 the RP cell line and **(E)** in the OPM-2 cell line. **(F)** Immunoblot of BLIMP1 and
 520 EZH2 expression following 24 h induction of RP cells expressing *NTmiR* or *PmiR1*
 521 treated with DMSO or 5 μ M MG-132 for 4h. **(G)** The ratio of EZH2 expression

relative to actin for RP *PmiR1* cells divided by RP *NTmiR* cells treated with DMSO or MG132 as in Fig. 2F. **(H)** Immunoblot of BLIMP1 and EZH2 expression following 48 h induction of RP *NTmiR* cells or *PmiR1* cells transduced with EGFP or EZH2-EGFP. **(I)** Percentage of live cells as determined by Trypan blue exclusion assay in the RP *PmiR1* cells transduced with EGFP or EZH2-EGFP. All p-values as determined by student's two-tailed t-test. (ns) $p > 0.05$; $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; $****p \leq 0.0001$. All graphs were plotted as the mean of three independent experiments with error bars representing standard deviation. **(J)** Immunoblot of histone extracts from RP cells stained for H3K27me3 with total H3 as a loading control following 48h tazemetostat treatment at the indicated concentrations. The 0 concentration was treated with vehicle control, DMSO.

533

Figure 3: BLIMP1 KD and EZH2 inhibition induce overlapping transcriptional changes

RNAseq results for **(A)** 48h-induced RP cells comparing *PmiR2* to *NTmiR*. Values are plotted as $\log_2$ fold change vs. $-\log_{10}(\text{q-value})$. Red indicates those genes with a q-value ≤ 0.05 and a $\log_2$ fold change ≤ -0.3 or ≥ 0.3 . Heat maps depicting the Z-score of the $\log_2$ fold change comparing *PmiR2* to *NTmiR* for three independent replicates looking at **(B)** B cell genes and **(C)** apoptosis genes. **(D)** RNAseq results for RP cells treated for 48h with 300nM tazemetostat compared to vehicle control (DMSO). **(E)** Overlapping genes with increased expression following *PRDM1* KD or tazemetostat treatment. **(F)** Overlapping genes with decreased expression following *PRDM1* KD or tazemetostat treatment. Overlaps tested using Fisher's exact test. **(G)** Overlaps of genes significantly induced by both BLIMP1 KD and tazemetostat with Hallmarks

546 gene sets from the molecular signatures database, showing the top 10 most
547 significantly overlapping gene sets.

548

549 **Figure 4:** BLIMP1 binds at a distance to the H3K27me3 mark

550 (A) Venn diagram of H3K27me3 and BLIMP1 peaks extended ± 500 bp, showing
551 overlaps in these regions. (ns) not significant as determined by hypergeometric test.
552 Called peaks determined by overlap from peak calling from two independent
553 experiments. (B) Enrichment of signal from ChIPseq tracks ± 3 kb from the centre of
554 BLIMP1 binding sites. Data depicts representative experiment of two biological
555 replicates. (C) Venn diagram of genes assigned to H3K27me3 and BLIMP1 peaks
556 showing overlapping genes. $p = 2.9 \times 10^{-25}$ as determined by Fisher's exact test. (D)
557 ChIPseq tracks for H3K27me3 and BLIMP1 in the RP cell line. Data represents
558 combination of reads from two independent experiments. (E) Enrichment of signal
559 from ChIPseq tracks ± 10 kb from the centre of BLIMP1 binding sites in the NCI-
560 H929 cell line. (F) Venn diagram depicting the overlap in genes with significantly
561 increased expression following BLIMP1 KD and genes assigned to BLIMP1 binding
562 sites. $p = 7 \times 10^{-15}$, as determined by Fisher's exact test. (G) Venn diagram depicting the
563 overlap in genes with significantly decreased expression following BLIMP1 KD and
564 genes assigned to BLIMP1 binding sites. (ns) not significant as determined by
565 Fisher's exact test. (H) Venn diagram depicting genes with significantly increased
566 expression following tazemetostat treatment overlapping with genes assigned to
567 H3K27me3 peaks. $p = 7.9 \times 10^{-9}$, as determined by Fisher's exact test. (I) Venn diagram
568 depicting genes with significantly decreased expression following tazemetostat
569 treatment overlapping with genes assigned to H3K27me3 peaks. (ns) not significant,

as determined by Fisher's exact test. All ChIPseq experiments were performed as two biological replicates.

572

Figure 5: BLIMP1 regulates a subset of target genes via EZH2

ChIPseq tracks for H3K27me3 and BLIMP1 over the genes (A) *ZFP36L1*, (B) *RCAN3*, (C) *TNFRSF14*, (D) *CIITA*, and (E) *TFEC*. (F) Bar graph depicting RT-qPCR experiments in RP cells expressing *NTmiR* or *PmiR1* with EGFP or EZH2-EGFP. The selected target genes bear peaks for either BLIMP1, H3K27me3 or both factors. All p-values as determined by student's two-tailed t-test. (ns) $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$. The bar graph was plotted as the mean of three independent experiments with error bars representing standard deviation.

581

Figure 6: BLIMP1 and EZH2 promote WM tumour immune evasion

(A) Heat maps showing z-score of the log₂ fold change for *PmiR2* compared to *NTmiR* and in RP cells with three independent replicates looking at genes involved in stimulation of T and NK cells, MHC molecules, inhibitory ligands and receptors, IFN and TNF receptors and downstream signalling. ChIPseq tracks for H3K27me3 and BLIMP1 in the RP cell line over (B) the immune surface molecule genes *HLA-B*, *MICA*, *LILRB1*, *NCR3LG1*, *TNFRSF1A*, or (C) the downstream signalling genes, *IFIT2* and *STAT1*. Data represents a combination of reads from two independent experiments. (D) Percentage CD56⁺LAMP1⁺ cells (Q2) representing degranulating NK cells, as determined by flow cytometry following co-culture with RP cells with *NTmiR* or *PmiR1*, or RP cells treated with DMSO or Tazemetostat. One representative experiment is displayed. (E) Relative quantification of the degranulation assay. (F) Cytotoxicity depicted in relative luminescence units (RLU),

as measured by adenylate kinase activity in the culture media following 4 h co-culture of NK cells with RP cells expressing *NTmiR* or *PmiR1*, or RP cells treated with DMSO or 1μM Tazemetostat. Cells were co-cultured at the effector:target ratio of 20:1. Results of the degranulation and cytotoxicity assays from four individual donors, performed in two pairs on two separate occasions. All p-values as determined by student's two-tailed t-test. (ns) $p > 0.05$; $*p \leq 0.05$; $**p \leq 0.01$; $****p \leq 0.0001$. All graphs were plotted as the mean of three independent experiments with error bars representing standard deviation.

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Figure 1

Figure 1: BLIMP1 promotes the survival of WM cells

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(A) BLIMP1 expression in the myeloma cell line OPM-2 and in WM cell lines RP, MW and BC as detected by immunofluorescence staining, with a bar graph representing percentage of BLIMP1bright cells. Scale bars represent 20µm. (B) Representative immunoblot of BLIMP1 expression following 48h induction of RP cells expressing NTmiR, PmiR1 or PmiR2. (C) Immunofluorescence staining of BLIMP1 following 48h induction in MW cells expressing NTmiR or PmiR1, with CellProfiler quantification. (D) Percentage of live cells as determined by Trypan blue exclusion assay in the RP and MW cell lines comparing PmiR1 or PmiR2 to NTmiR following 48h of induction. (E) Immunoblot depicting lentiviral ectopic expression of EGFP or BLIMP1-EGFP in the RP PmiR1 cells, next to the RP NTmiR cells. (F) The percentage of live RP PmiR1 cells with ectopic EGFP or BLIMP1-EGFP expression determined by the Trypan blue exclusion assay normalised to the percentage of live cells following transduction of RP NTmiR cells with EGFP or BLIMP1-EGFP. BLIMP1-EGFP compared to EGFP at Day 2. (G) Per cent reduction as measured by resazurin assay for RP PmiR1 cells with EGFP compared to BLIMP1-EGFP, five days after miR induction. All p-values as determined by student's two-tailed t-test. *p ≤ 0.05; **p ≤ 0.01; *** p ≤ 0.001; ****p ≤ 0.0001. All graphs were plotted as the mean of three independent experiments, with error bars representing standard deviation.

Figure 2

Figure 2: BLIMP1 maintains EZH2 protein levels in WM cells

(A) Immunoblot of BLIMP1 and EZH2 expression following 48h induction of RP cells expressing NTmiR, PmiR1 or PmiR2. (B) Immunoblot of BLIMP1 and EZH2 expression following 48h induction of OPM-2 cells expressing NTmiR compared to PmiR1. (C) Immunofluorescence staining of BLIMP1 and EZH2 expression following 48 h induction of RP cells expressing NTmiR or PmiR1, transduced with EGFP or BLIMP1-EGFP. Scale bars represent 20 μ m. (D) RT-qPCR results depicting relative mRNA expression of PRDM1 and EZH2 normalised to PPIA and ACTB in the RP cell line and (E) in the OPM-2 cell line. (F) Immunoblot of BLIMP1 and EZH2 expression following 24 h induction of RP cells expressing NTmiR or PmiR1 treated with DMSO or 5 μ M MG-132 for 4h. (G) The ratio of EZH2 expression relative to actin for RP PmiR1 cells divided by RP NTmiR cells treated with DMSO or MG132 as in Fig. 2F. (H) Immunoblot of BLIMP1 and EZH2 expression following 48 h induction of RP NTmiR cells or PmiR1 cells transduced with EGFP or EZH2-EGFP. (I) Percentage of live cells as determined by Trypan blue exclusion assay in the RP PmiR1 cells transduced with EGFP or EZH2-EGFP. All p-values as determined by student's two-tailed t-test. (ns) $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. All graphs were plotted as the mean of three independent experiments with error bars representing standard deviation. (J) Immunoblot of histone extracts from RP cells stained for H3K27me3 with total H3 as a loading control following 48h tazemetostat treatment at the indicated concentrations. The 0 concentration was treated with vehicle control, DMSO.

Figure 3: BLIMP1 KD and EZH2 inhibition induce overlapping transcriptional changes

RNAseq results for (A) 48h-induced RP cells comparing PmiR2 to NTmiR. Values are plotted as log2 fold change vs. $-\log_{10}(\text{q-value})$. Red indicates those genes with a q-value ≤ 0.05 and a log2 fold change ≤ -0.3 or ≥ 0.3 . Heat maps depicting the Z-score of the log2 fold change comparing PmiR2 to NTmiR for three independent replicates looking at (B) B cell genes and (C) apoptosis genes. (D) RNAseq results for RP cells treated for 48h with 300nM tazemetostat compared to vehicle control (DMSO). (E) Overlapping genes with increased expression following PRDM1 KD or tazemetostat treatment. (F) Overlapping genes with decreased expression following PRDM1 KD or tazemetostat treatment. Overlaps tested using Fisher's exact test. (G) Overlaps of genes significantly induced by both BLIMP1 KD and tazemetostat with Hallmarks gene sets from the molecular signatures database, showing the top 10 most significantly overlapping gene sets.

Figure 4

Figure 4: BLIMP1 binds at a distance to the H3K27me3 mark

(A) Venn diagram of H3K27me3 and BLIMP1 peaks extended ± 500 bp, showing overlaps in these regions. (ns) not significant as determined by hypergeometric test. Called peaks determined by overlap from peak calling from two independent experiments. (B) Enrichment of signal from ChIPseq tracks ± 3 kb from the centre of BLIMP1 binding sites. Data depicts representative experiment of two biological replicates. (C) Venn diagram of genes assigned to H3K27me3 and BLIMP1 peaks showing overlapping genes. $p = 2.9e-25$ as determined by Fisher's exact test. (D) ChIPseq tracks for H3K27me3 and BLIMP1 in the RP cell line. Data represents combination of reads from two independent experiments. (E) Enrichment of signal from ChIPseq tracks ± 10 kb from the centre of BLIMP1 binding sites in the NCI-H929 cell line. (F) Venn diagram depicting the overlap in genes with significantly increased expression following BLIMP1 KD and genes assigned to BLIMP1 binding sites. $p = 7e-15$, as determined by Fisher's exact test. (G) Venn diagram depicting the overlap in genes with significantly decreased expression following BLIMP1 KD and genes assigned to BLIMP1 binding sites. (ns) not significant as determined by Fisher's exact test. (H) Venn diagram depicting genes with significantly increased expression following tazemetostat treatment overlapping with genes assigned to H3K27me3 peaks. $p = 7.9e-9$, as determined by Fisher's exact test. (I) Venn diagram depicting genes with significantly decreased expression following tazemetostat treatment overlapping with genes assigned to H3K27me3 peaks. (ns) not significant, as determined by Fisher's exact test. All ChIPseq experiments were performed as two biological replicates.

Figure 5

Figure 5: BLIMP1 regulates a subset of target genes via EZH2

ChIPseq tracks for H3K27me3 and BLIMP1 over the genes (A) ZFP36L1, (B) RCAN3, (C) TNFRSF14, (D) CIITA, and (E) TFEC. (F) Bar graph depicting RT-qPCR experiments in RP cells expressing NTmiR or PmiR1 with EGFP or EZH2-EGFP. The selected target genes bear peaks for either BLIMP1, H3K27me3 or both factors. All p-values as determined by student's two-tailed t-test. (ns) $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$. The bar graph was plotted as the mean of three independent experiments with error bars representing standard deviation.

Figure 6

Figure 6: BLIMP1 and EZH2 promote WM tumour immune evasion

(A) Heat maps showing z-score of the log2 fold change for PmiR2 compared to NTmiR and in RP cells with three independent replicates looking at genes involved in stimulation of T and NK cells, MHC molecules, inhibitory ligands and receptors, IFN and TNF receptors and downstream signalling. ChIPseq tracks for H3K27me3 and BLIMP1 in the RP cell line over (B) the immune surface molecule genes HLA-B, MICA, LILRB1, NCR3LG1, TNFRSF1A, or (C) the downstream signalling genes, IFIT2 and STAT1. Data represents a combination of reads from two independent experiments. (D) Percentage CD56+LAMP1+ cells (Q2) representing degranulating NK cells, as determined by flow cytometry following co-culture with RP cells with NTmiR or PmiR1, or RP cells treated with DMSO or Tazemetostat. One representative experiment is displayed. (E) Relative quantification of the degranulation assay. (F) Cytotoxicity depicted in relative luminescence units (RLU), as measured by adenylate kinase activity in the culture media following 4 h co-culture of NK cells with RP cells expressing NTmiR or PmiR1, or RP cells treated with DMSO or 1µM Tazemetostat. Cells were co-cultured at the effector:target ratio of 20:1. Results of the degranulation and cytotoxicity assays from four individual donors, performed in two pairs on two separate occasions. All p-values as determined by student's two-tailed t-test. (ns) $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$. All graphs were plotted as the mean of three independent experiments with error bars representing standard deviation.