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1 **Pan-HDAC inhibitors restore PRDM1 response to IL-21 in CREBBP mutated**
2 **follicular lymphoma**

3
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36
37 In a number of Phase I/II clinical trials, HDACi therapies have shown clinical
38 responses including complete remissions in previously multi-treated follicular
39 lymphoma (FL) patients. Even if multiple biological effects have been described for
40 these drugs no effect on the master plasma cell regulator gene, *PRDM1* has been so
41 far described in the context of the FL. Indeed, our study revealed that FLs with a
42 CREBBP loss of function (concerns more than 50% of FLs) were unable to
43 upregulate *PRDM1* expression despite the presence of IL-21 in the tumor
44 microenvironment, a potent inductor of *PRDM1*. In this context we found that pan-
45 HDACi can restore *PRDM1* expression as well as other plasma cell genes, indicating
46 a possible re-initiation of FL B cells differentiation. Our results highlight one effect of
47 pan-HDACis to overcome FLs differentiation blockade.

48 **Abstract**

49 Purpose: Follicular lymphoma (FL) arises from a germinal center B-cell proliferation
50 supported by a bidirectional crosstalk with tumor microenvironment, in particular with
51 follicular helper T cells (Tfh). We explored the relation that exist between the
52 differentiation arrest of FL cells and loss-of-function of CREBBP acetyltransferase.

53 Experimental design: The study used human primary cells obtained from either, FL
54 tumors characterized for somatic mutations, or inflamed tonsils for normal germinal
55 center B cells. Transcriptome and functional analyses were done to decipher the B
56 and T cell crosstalk. Responses assessed by flow cytometry and molecular biology
57 including ChIP-qPCR approaches.

58 Results: Conversely to normal B cells, FL cells are unable to upregulate the
59 transcription repressor PRDM1, required for plasma cell differentiation. This defect
60 occurs although the FL microenvironment is enriched in the potent inducer of PRDM1,
61 IL-21, highly produced by Tfh. In FL carrying CREBBP loss-of-function mutations,
62 we found a lack of IL-21-mediated PRDM1 response associated with an abnormal
63 increased enrichment of the BCL6 protein repressor in *PRDM1* gene. Moreover, in
64 these FL cells, pan-HDAC inhibitor Vorinostat restored their PRDM1 response to IL-
65 21 by lowering BCL6 bound to *PRDM1*. This finding was reinforced by our
66 exploration of FL patients treated with another pan-HDAC inhibitor. Patients showed
67 an increase of plasma cell-identity genes, mainly *PRDM1* and *XBP1*, which underline
68 the progression of FL B cells in the differentiation process.

69 Conclusion: Our data uncover a new mechanism by which pan-HDAC inhibitors may
70 act positively to treat FL patients through the induction of the expression of plasma
71 cell genes.

72 **Introduction**

73 Adaptive immune responses involve the formation of germinal centers (GCs), which
74 are specialized structures allowing differentiation of high affinity B cells into long-lived
75 memory B cells and plasma cells (PCs). PCs are generated through specific
76 transcriptional programs influenced by numerous signals delivered by the
77 microenvironment including IL-21. This latter is a potent inducer of PCs and is
78 produced by GC CD4⁺ helper T cells called follicular helper T cells (Tfh) (1). Akin to
79 most cytokines, IL-21 activates mainly JAK3/STAT3 signaling pathways (2) known to
80 play a highly selective role in PC differentiation (3) and apoptosis depending on the
81 context (4). In humans, blockade of IL-21 inhibits PC generation. Tfh interacting with
82 IL-21 receptor-expressing GC B cells could thus impact B cell destiny via the delivery
83 of IL-21 signal (3,5). Human PCs emerging through IL-21/pSTAT3 signaling
84 enhancement has concomitantly up-regulated *PRDM1/BLIMP1* and down-regulated
85 *BCL6* gene expression (6,7). *PRDM1/BLIMP1* is considered as the master PC factor
86 that antagonizes *BCL6* which one sustains B cell identity (8,9). Therefore,
87 *PRDM1/BLIMP1* and *BCL6* mutually inhibit each other and *BCL6*-related inhibition of
88 PC generation is predominantly related to *BCL6* binding at intron 3 of *PRDM1* where
89 *BCL6* recruits MTA3, which acts as a co-repressor (10). Moreover this *PRDM1*
90 intronic region contains an enhancer bound by CREBBP (11). CREBBP acts as a
91 transcriptional coactivator of many different transcription factors through its intrinsic
92 acetylation function on histone but also on non-histone proteins including *BCL6*.
93 Indeed, CREBBP binds and acetylates *BCL6* leading to the inactivation of its
94 transcriptional repressor function (12,13).

95 In follicular lymphoma (FL), we demonstrated earlier that the tumor microenvironment
96 is enriched for Tfh cells which ones sustain neoplastic cells (14,15). It is generally
97 accepted in the field that FLs are tumors reflective of centrocytes that fail to
98 differentiate beyond the GC exit point. Purified FL B cells compared to normal GC B
99 cells does not identify a radically opposed signature but rather substantial
100 modification of normal GC expression driving most likely by numerous genetic and
101 epigenetic somatic modifications (14,16-18). Both histone acetyl transferases
102 CREBBP and EP300 are commonly mutated in FL and *CREBBP* loss-of-function
103 affects preferentially H3K27 acetylation which depletion lead to downregulation of
104 genes involved in GC output (19). Interestingly, the expression of these genes can be
105 restored after inhibition of HDAC3 (20).

106 In this study we assessed *PRDM1* gene expression and regulation in order to
107 achieve new insights on the differentiation blockade that characterizes the FL. We
108 showed that despite a functional capacity to activate the IL-21/pSTAT3 signaling,
109 nonfunctional CREBBP FL cells were unable to increase *PRDM1* expression. In
110 these FLs, induction of PRDM1 in response to IL-21 could be restored through the
111 use of pan-HDAC inhibitors.

112

113 **Material and Methods**

114 *Samples*

115 Subjects were recruited under institutional review board approval and informed
116 consent process (French Minister authorization DC-2016-2565). An informed written
117 consent was obtained from each subject or subject's guardian. The study was
118 conducted in accordance of the Declaration of Helsinki. Normal GC-derived B and T

119 cells were isolated from human tonsils and reactive lymph nodes. FL tumors were
120 obtained from patients that underwent a surgical biopsy during a diagnosis procedure
121 and from patients with refractory/relapsed FL recruited during the phase I/II study
122 based on an oral pan-HDACi drug (21).

123

124 *Transcriptomic data*

125 For transcriptomic analysis, we used highly purified B lymphocyte fractions that were
126 sorted using combinations of monoclonal antibodies and FACSARIA (BD
127 Biosciences) system. Extracted RNAs were hybridized on an Affymetrix Human
128 Genome U133 Plus 2.0 Array and strategy for raw data normalization and filtering is
129 detailed in supplementary methods.

130

131 *Gene expression analysis*

132 The relative quantification of gene expression was determined using the $2^{-\Delta\Delta Ct}$
133 method, then normalized to at least an internal control gene (*ABL1*, *GAPDH* and/ or
134 *HPRT1*) and relative to a calibrator control sample corresponding to a mix of cDNA of
135 peripheral blood mononuclear cells (PBMC) from several healthy donors. Statistical
136 analysis using GraphPad Prism Software used Mann-Whitney test.

137

138 *Flow cytometry analysis, tissue immunostaining, western blotting, and FISH analysis*

139 All antibodies and technical details concerning protein expression and FISH analyses
140 are in supplementary methods, Table S4 and Supplementary Figures S3, S4 & S8.

141

142 *Culture conditions and molecular analyses*

143 Cells were cultured for 2h before receiving indicated treatments for 24 hours, then
144 CD19/CD20 viable B cells were collected and used for subsequent DNA and RNA
145 extraction. Detailed procedures are given in supplementary methods.

146

147 *Somatic mutations assessment*

148 We performed the SureSelect targeted-capture strategy from Agilent Technologies
149 (Courtaboeuf, France) using a panel of 34 genes described previously (22) with
150 subsequent paired end sequencing on an Illumina HiSeq 1500 Platform (Illumina,
151 San Diego, USA). For variants detection SureCall software from Agilent technologies
152 was used (see Supplementary methods, Tables S2B and S3B).

153

154 *Statistical analyses*

155 Statistical analyses were performed with the GraphPad Prism software V5
156 (GraphPad Software, San Diego, CA, USA) as indicated in supplemental methods
157 section.

158

159 **Results**

160 **Follicular lymphoma B cells are functionally unable to regulate genes involved** 161 **in plasma cell differentiation**

162 B cell differentiation process is supported by the microenvironment through the
163 delivery of soluble and membrane signals (23,24). Among them, CD40L and IL-21
164 are major contributors to the transcriptional emergence of PC-identity genes in
165 committed B cells (3,7,25). To decipher specific CD40L and IL-21 targeted genes,

166 freshly purified CBs were cultured for 3h in presence or not of CD40L and IL-21
167 before transcriptome analysis were performed. We found a total of 4,932 genes
168 differentially expressed with 2,154 up- and 2,778 down-regulated genes between
169 unstimulated and stimulated conditions (FDR<0.05) (Figure 1A). In particular,
170 *PRDM1* expression is increased 3.5 times upon CD40L and IL-21 stimulation
171 ($P=0.001$) (Figure 1B). In parallel, using the same approach, we analyzed highly
172 purified human centroblastic (CB) and centrocytic (CC) GC-derived B cells from
173 tonsils and highly purified FL B cells. At first, a total of 4,654 genes (FDR<0.05) with
174 2,347 up- and 2,307 down-regulated genes were found differentially expressed
175 between centroblasts (CBs) and centrocytes (CCs) (Figure 1C-D). We display list
176 comparisons using Venn diagrams in order to find which subsets of genes are
177 involved in both CB/CC transition and CB response to IL-21/CD40L signaling. 804
178 up- and 965 down-regulated genes (total of 1,769 genes) were common to both lists
179 (Figure 1C) and major hallmark pathways associated with these genes were related
180 to immune responses and cell proliferation respectively (Supplementary Table S1).
181 We next applied this 1,769-gene signature to compare CB, CC and FL B cell
182 transcriptomes. Unsupervised hierarchical analysis clustered FL B cells separately
183 from other cells with in particular an opposite pattern of expression compared to CBs
184 (Figures 1D). We delimited two boxes of probesets based on the clustering and
185 found that probesets positively expressed in Box1 were vastly (>95%) connected to
186 CBs while the Box2-positive probesets were associated to FL B cells (>82%). In
187 addition, Box2 contained 95.8% of the 804 genes upregulated in the 1,769-gene
188 signature. Interestingly, in this analysis the CCs presented somewhere an
189 intermediate position suggesting a polarized axis where FL cells occupy the most

190 advanced position in the B cell differentiation (Figure 1E). To complete our
191 characterization, we use Ingenuity Pathways Analysis (IPA, Ingenuity® Systems,
192 www.ingenuity.com) and focused on previously described molecular pathways
193 enriched in B cells and PCs (26). Globally, we confirmed that Box1 genes are related
194 to CB signatures (cell cycle and FOXM1 transcription factor network), while Box2
195 presented plasmablast and early PC features (Figure 1F). In this context, we noticed
196 with interest the strong repression of *PRDM1* in FLs (highlighted in Figure 1D). This
197 finding was confirmed using an independent cohort of 23 FLs previously explored
198 and where total CD19+ B cells were compared to B cells issued from reactive lymph
199 nodes (Figure 1G) (14). Immunohistochemistry for BLIMP1 protein showed a totally
200 negative staining in FL while normal GCs showed very weak expression and tumor
201 plasmacytoma a strong positivity (Supplementary Figure S1C). Our transcriptome
202 findings in FL are in contradiction with the transcriptional network of normal B cells
203 (27) and also with our previous data (28) on the hierarchical clustering showing a
204 transcriptional switch between CB and CC subtypes. Altogether our data suggest the
205 existence of a deregulation of the transcriptional *BCL6/PRDM1* balance in FLs.
206 Indeed, FLs maintain the expression of B cell identity genes including *BACH2* and
207 *BCL6* (*PAX5* is missing in our 1,769-gene signature) besides low *PRDM1* expression,
208 while on the other hand these cells express *IRF4* and *XBP1*, both tightly connected
209 to the PC identity. By IPA Ingenuity® we found that the Box2 was significantly
210 enriched for the CD40L/CD40 pathway (Supplementary Figure S1A). Gene set
211 enrichment assays (GSEA) applied on the 1,769-gene signature identified a
212 significant enrichment in FLs compared to CB for CD40L, IL-21, and plasma cells up-
213 regulated genes signatures (Figure S1B). Collectively, our results evidence that FL B

214 cells are most likely blocked at a terminal B-cell differentiation step characterized by
215 a low expression of *PRDM1* despite the presence of substantial CD40L and IL-21
216 microenvironment signaling.

217

218 **Impaired PRDM1 response after IL-21 stimulation in FL**

219 During the differentiation of CCs into PCs, the upregulation of *PRDM1* through the IL-
220 21 signaling was previously demonstrated in normal and lymphoma B cells (4,29).
221 Based on these data and for reasons of sample saving in order to complete our study
222 on primary FL cells, we decided to focus our investigations solely on IL-21 signaling.
223 Highly purified FL-B cells of 18 patients (clinical details are in Supplementary Table
224 S2A) were cultured for 24h in presence or not of IL-21 and cell viability was
225 systematically monitored (Supplementary Figure S3). As control, we used L3055
226 centroblastic cell line (named hereafter, control L3055), an EBV-negative Burkitt
227 lymphoma cell line phenotypically and functionally similar to normal CBs (30) carrying
228 besides the rearranged *MYC* locus no additional alterations affecting *BCL6* and
229 *PRDM1* loci as shown by CGH array (Supplementary Figure S2). Control L3055
230 increased significantly *PRDM1* expression upon IL-21 without effect on *BCL6* (Figure
231 2A & B). Protein BLIMP1/*PRDM1* was detectable by flow cytometry for control tonsil-
232 derived GC B cells and by western blot for L3055 cells after 24h of IL21 stimulation
233 (Supplementary Figure S2). In contrast, the 18 FLs did not significantly modify their
234 expression of *PRDM1* and *BCL6* (Figure 2A & B; Supplementary Figure S3C-D).
235 Spearman's analysis to find a correlation between the expression of PRDM1 and
236 BCL6, showed that a large majority of FLs did not respond for both genes after IL21

237 stimulation (Figure 2C). These results are in line with our transcriptome analysis
238 findings and confirm that most FL B cells exhibit a defect in *PRDM1* response.

239

240 **FL Tfh_s and FL B cells show a functional and increased IL-21/pSTAT3**
241 **response**

242 We used total cell suspensions from FL tumors to evaluate the functional capacities
243 of B-cells and Tfh_s. Control cell counterparts came from non-malignant tonsils or
244 reactive lymph nodes (rLNs). The gating strategy of the flow cytometry analysis
245 defined a specific CD3/CD4/CxCR5/PD-1-positive cell population corresponding to
246 Tfh_s (Supplemental Figure S4). As previously described, Tfh_s increased in number in
247 FLs compared to rLNs (Figure 3A) (14,31). Functional experiments on Tfh_s showed
248 an enhanced production of IL-21 after stimulation by PMA-ionomycin (Figure 3B)(32)
249 as well as a significant increase of the pSTAT3 response after 10 min of IL-21
250 (Figure 3C). FL B cells from 20 patients showed after IL-21 stimulation a significant
251 increase in pSTAT3 compared to controls. Interestingly this tonic response was
252 detected in malignant and nonmalignant B cells, both discriminated with BCL2
253 intracellular staining (Figure 3D and Supplemental Figure S4). In addition, both B cell
254 populations for the same FL increased proportionally their pSTAT3 expression
255 (Figure 3E). Moreover, 24h of IL-21 stimulation led to the expression of *BATF*, a
256 specific pSTAT3-target (33) (Figure 3F). We completed our analysis by evaluating
257 the expression of pSTAT3 by immunohistochemistry in 7 FLs and compared the
258 results with 2 rLNs. In normal GC, B cells presented a pSTAT3 staining mainly in
259 medium and large B cells localized within the light zone of the GC in the vicinity of

260 PD1-positive T cells (Left Panel, Figure 3G). In FL, this pattern of expression was lost
261 and pSTAT3-positive B cells were more numerous and formed clusters (Right panel,
262 Figure 3G). Overall, we found a mean number of 40 pSTAT3-positive cells per GC in
263 FL (ranged from 29 to 51) compared to a mean of 19 in rLNs. Unlike rLNs, some FL
264 Tfh_s were positive for pSTAT3 in agreement with our above flow cytometry data
265 (Right Panel, Figure 3G). Altogether, our data showed that FLs present an enhanced
266 and functional IL-21/pSTAT3 signaling.

267

268 **Nonfunctional CREBBP FLs increased BCL6 binding to *PRDM1* gene in**
269 **response to IL-21**

270 Genetic alterations of *CREBBP* may alter its acetyltransferase activity, which may
271 abolish BCL6 acetylation and maintain its repressor activity on *PRDM1* (10,12,13).
272 We next sought for somatic mutations in *CREBBP* coding region included in our 34-
273 genes lymphopanel beside other chromatin modifier genes (*EP300*, *EZH2*, *KMT2D*,
274 and *MEF2B*) (34) used for capture-targeted deep-sequencing strategy. Fourteen FLs
275 out of 20 were mutated for *CREBBP* at least once (70%) including 10 non-
276 synonymous variants (SNV) located in the HAT catalytic domain (Histone
277 acetyltransferase domain), 2 SNV outside the HAT and 2 frameshift (fs) in the N-
278 terminal region. For 12 of these variants (HAT located and fs) a nonfunctional
279 CREBBP protein is encoded (Figure 4A and Supplementary Table S2C) (12). *EP300*
280 (another gene with a HAT domain) was mutated in 2 cases but outside HAT domain
281 (Supplementary Table S2C). Altogether, 12 out of 20 FLs presented a functional loss
282 of *CREBBP* owing to genetic alterations.

283 To determine the binding capacity of BCL6 protein to intron 3 (INT3) of *PRDM1* we
284 used a chromatin immunoprecipitation (ChIP) approach followed by Q-PCR (see
285 procedure details in Supplementary Figure S5). Highly purified FL-B cells and control
286 L3055 were cultured for 24h in the presence or not of IL-21. BCL6 binding to *PRDM1*
287 was decreased in L3055 upon IL-21, and correlates with its increased expression
288 due to a probable releasing BCL6-mediated transcriptional repression (Figure 4B).
289 We then explored 10 FLs out of 18 previously explored for *PRDM1* expression
290 (Figure 2A), for which we had sufficient viable B cells for ChIP assays. For 2
291 (FL_6108 and FL_5511) out of 4 wild type FLs for *CREBBP*, the IL-21-induced
292 *PRDM1* expression was associated with a decrease of BCL6 enrichment at INT3 of
293 *PRDM1*, thus behaving like control L3055. Six other FLs, characterized by a positive-
294 BCL6 protein immunohistochemistry staining and a loss-of-function variant of
295 *CREBBP*, we observed a strong enrichment of the BCL6 binding except for FL_5008.
296 This finding correlated with the absence of IL-21 induction of *PRDM1* gene
297 expression and data indicate that in response to IL21 stimulation the tumor cells
298 operate an active silencing of the *PRDM1* gene, possibly facilitating local recruitment
299 of the BCL6 repressor (Figure 4B). Globally, our data suggest that a link exist
300 between BCL6 binding, *PRDM1* response to IL-21, and the functional activity of
301 *CREBBP*. Therefore we decided to explore further the binding of BCL6 protein to
302 INT3 of *PRDM1* in response to IL-21 with the use of a histone acetyltransferase
303 inhibitor (HDACi).

304

305 **Pan-HDAC inhibitor vorinostat restores *PRDM1* response to IL-21 in**
306 **nonfunctional CREBBP FLs**

307 We postulated that CREBBP-mutated FLs might present a diminished acetylated
308 form of BCL6 protein conferring a potential oncogenic activity (12,13). Thus, we
309 hypothesized that by restoring acetylation state of histones and proteins in FL B cells
310 using an HDAC inhibitor, we would be able to restore *PRDM1* response to IL-21. We
311 used vorinostat, also known as suberanilohydroxamic acid (SAHA), a potent pan-
312 HDACi, on 6 CREBBP-mutated FLs and on control L3055. Purified cells were
313 analyzed after 24h of culture using three different conditions: IL-21 alone, IL-21 plus
314 vorinostat, and vorinostat alone. None of these conditions triggered cell death
315 (Supplementary Figure S7). Addition of SAHA to IL21 induced a significant increase
316 of BLIMP1/*PRDM1* mRNA and protein expression without modification of BCL6
317 binding to *PRDM1* gene (Figure 5 and Supplementary Figure S6). For 4 out of 6 FLs,
318 *PRDM1* expression increased under IL-21 plus vorinostat compared to IL-21 alone
319 and was associated with a *BCL6* expression decrease (Figure 5A-B) leading overall
320 to a significant positive effect on the *PRDM1/BCL6* expression balance as shown for
321 the control L3055 ($P=0.041$; Figure 5D). In addition, we detected for all 6 FLs a clear
322 BCL6 occupancy decrease to the INT3 of *PRDM1* under IL-21 plus vorinostat (Figure
323 5C). The vorinostat-only condition compared to IL-21 alone did not significantly
324 increase the *PRDM1/BCL6* ratio ($P=0.93$; Figure 5D) while a decrease of BCL6
325 binding occurred in five out of six cases (Figure 5C-D). In conclusion, our
326 observations could be summarized schematically using a scale where SAHA addition
327 to IL-21 will reverse the abnormal *BCL6/PRDM1* transcriptional gene expression

328 equilibrium in FLs by increasing *PRDM1* while decreasing *BCL6* expression (Figure
329 5E).

330

331 **Increased expression of PC-related genes in FL patients treated with a new**
332 **pan-HDACi**

333 To determine if HDACi therapy in FL may affect the expression of PC master genes,
334 we analyzed 4 out of 7 FL patients included in a multicenter phase I/II study testing a
335 new pan-HDACi in refractory/relapsed B-cell lymphoproliferative disease (21).
336 Somatic mutations screening was performed on tumor DNA at diagnosis and when
337 patients re-progressed (Supplementary Table S3). FL_CAN patient still in complete
338 remission 5 years after the inclusion (the drug discontinued after 4 years) was
339 *CREBBP* wild-type at diagnosis but mutated in the HAT domain of *EP300*, which
340 encoded a protein that, like *CREBBP*, sustains acetylation-mediated inactivation of
341 *BCL6* repressive activity (12) (Figure 6A). For patients FL_CON and FL_DAD we
342 detected non-synonymous variants within, respectively, the HAT domain of *EP300* or
343 *CREBBP* before and after treatment (Figure 6A). These two patients, one with 7
344 months of stable disease (FL_DAD) and the other without response to the drug
345 (FL_CON), presented a clonal evolution in their re-biopsy (Figure 6A), probably
346 leading to the therapeutic resistance. For FL_CAS patient who had an objective 28-
347 month response to the drug before relapse, he was not mutated for *CREBBP* or
348 *EP300*. However, he showed also a clonal evolution upon treatment and notably the
349 loss of the CD79B mutation, which plays a role in antigenic stimulation in chronic
350 active BCR signaling (Figure 6A).

351 Our investigations on tumor RNA extracts showed globally an increase in the
352 expression of the 3 tested PC transcription factors, *PRDM1*, *IRF4* and *XBP1* in re-
353 biopsies compared to the initial tissues while B cell-identity factors remain stable
354 except for *BCL6*, and *BACH2* in patient FL_CON only (Figure 6B). The expression of
355 *PRDM1* in FL_CON and FL_CAS increased during treatment whereas FL_DAD
356 showed stable expression but had a higher level of *PRDM1* expression at the time of
357 inclusion compared to other patients. In addition, FL_DAD was the only one who
358 showed a marked increase in the expression of *IRF4*. The 3 patients for whom the
359 treatment failed showed a clear increase of *XBP1* expression. Interestingly, for
360 patients FL_DAD and FL_CON, we observed an elevation of the expression of the
361 active form of *XBP1*, *XBP1s*, associated with a clear up-regulation of the UPR
362 (unfolding protein response) sensor *ERN1*, a gene encoding the endoplasmic
363 reticulum kinase involved in *XBP1* maturation (Figure 6C) (35). These mechanisms
364 are essential for PC differentiation (36). Overall, increased expressions of key PC-
365 identity genes under pan-HDACi treatment suggest that FLs underwent some
366 differentiation but that tumor cells still escape the drug (Figure 5D).

367

368 Discussion

369 In 2006, two different groups (37,38) described the presence of *PRDM1*/BLIMP1
370 inactivation in 50% of non-GC DLBCL owing to alterations on both alleles by either
371 deletion or mutations. This loss of function is critical for the lymphomagenesis
372 process giving to BLIMP1 the role of a *bona fide* tumor suppressor. This inactivation
373 is mutually exclusive with *BCL6* alterations (39). However, they also identified few

374 non-GC DLBCLs without *PRDM1* genetic alterations but with a lack of BLIMP1
375 protein, suggesting that other mechanisms could be responsible for *PRDM1*/BLIMP1
376 inactivation. In our study, we identified in FL B cells compared to normal counterparts
377 a downregulation of *PRDM1* expression while our screening for somatic mutations
378 rule out the existence of *PRDM1* genetic alterations. Our transcriptome analysis of
379 normal GC B cells subjected to IL-21/CD40L stimuli identified a specific signature
380 representative of the Tfh-delivery signal promoting terminal B-cell differentiation; *i.e.*
381 upregulation of PC-identity genes including *PRDM1* associated to downregulation of
382 B cell-identity genes (6,7). In comparison, the IL-21/CD40L signature in FLs
383 transcriptome is reversed with upregulation of some PC-identity genes (*e.g.*, *IRF4*
384 and *XBP1*) and downregulation of *PRDM1*, which gene expression is mandatory for
385 PC maturation and maintenance (40,41). Therefore, our observations that FL cells
386 are low *PRDM1* expressers suggest a blockade of FL cells maturation that might be
387 due to the impossibility of tumor cells to express *PRDM1*/BLIMP1.

388 Studies human and mouse models revealed that IL-21 plays a crucial role in the
389 development of B cell immunoglobulin responses through the induction of *PRDM1*
390 expression (42,43). Our functional experiments in control L3055 found a positive IL-
391 21-mediated *PRDM1* response whilst FL cells showed low baseline expression of
392 *PRDM1* and a lack of induction with IL-21. This finding suggested that FL cells take
393 advantage of the GC's "fertile soil" to develop and initiate the terminal differentiation,
394 however the process is stopped in a step of differentiation characterized by their
395 inability to positively regulate the expression of *PRDM1* in absence of any genetic
396 alteration of this gene (44). In chronic lymphocytic leukemia, Duckworth *et al.*
397 described a transcriptional repression of *PRDM1* upon IL-21 owing to the loss of

398 chromatin active marks (45). In some aggressive lymphomas, silencing of *PRDM1*
399 was related to DNA hypermethylation in regulatory regions of the gene (46). In our
400 study we investigated the proximal promoter and intron 3 of the gene and found very
401 low level of methylation ruling out this mechanism (data not shown).

402 The IL-21 signaling goes mostly through a potent induction of the STAT3 pathway
403 involving the binding to a pSTAT3-IRF4 consensus site of *PRDM1* leading to the
404 gene upregulation (2,29). Herein, we confirmed previous descriptions in FL with the
405 presence of a Tfh-enriched microenvironment (14), an enhanced capacity of Tfh cells to
406 secrete IL-21 (15) and the increased proportion of both, FL B cells and Tfh cells to
407 produce pSTAT3 in response to IL-21 compared to their normal counterparts. These
408 findings may reflect the existence of a chronically active stimulation in FL
409 microenvironment leading to a prompt pSTAT3 functional response. Indeed, we
410 found an enhanced expression of *BATF*, a target gene of pSTAT3, after IL-21
411 stimulation and the presence of pSTAT3-positive cell clusters in tumor tissues.

412 Both, *BCL6* and *BLIMP1* are known to negatively cross-regulate each other.
413 Therefore, during B cell differentiation, crossing the restriction point thanks to a
414 reverse transcriptional balance (*i.e.* *PRDM1* goes up while *BCL6* decreases) will be
415 crucial to complete the final cell commitment (6,47). The role played by IL-21 in this
416 control has been little explored mainly because of the absence of reliable B cell lines.
417 Our cell culture experiments on control L3055 showed that the IL-21-mediated
418 *PRDM1* upregulation was associated with a decrease of *BCL6* occupancy on
419 *PRDM1*. For primary cultured FL B cells only 2 cases presented similar results while
420 the other 8 showed a decrease in their *PRDM1* expression by IL-21 and, in parallel,

421 they increased the binding of BCL6 protein to *PRDM1*. Among them 6 FLs had a
422 functional loss of CREBBP, which has an impact on the BCL6 acetylation status.
423 Indeed, in normal cells, CREBBP binds and acetylates BCL6 which disrupts its ability
424 to recruit histone deacetylases thereby enhancing its capacity to repress transcription
425 of target genes, such as *PRDM1* (12,13). We therefore speculate that HDACi
426 treatments could lead to accumulation of inactive acetylated BCL6, cell cycle arrest,
427 and apoptosis in B cell lymphoma cells (13). Our experiments with vorinostat on
428 nonfunctional CREBBP FL cells showed globally an increase in *PRDM1* expression
429 after IL-21 exposure. This gain of PRDM1 response was associated to a decrease of
430 BCL6 protein occupancy on INT3 of *PRDM1* and a decrease in *BCL6* gene
431 expression leading indirectly to an enhanced PRDM1 functional response through
432 the PRDM1/BCL6 balance, which one drives the B cell terminal differentiation (26). It
433 is of interest to notice that FL cases wild type for CREBBP maintained a positive
434 *PRDM1* response to IL-21 while 3 out of 4 presented a deregulated *BCL6* gene
435 (3q27 positive) with limited modifications of BCL6 binding to *PRDM1* (Figure 4). The
436 fourth case wild type for CREBBP and without 3q27 abnormality, responded to IL-21
437 like the control L3055, *i.e.* a positive PRDM1 response to IL-21 accompanied with a
438 decrease of BCL6 enrichment at INT3 of *PRDM1*. Taken altogether these findings,
439 and despite the lack of protein verification on FL cells due to material scarcity, we
440 speculate that the decrease of BCL6 binding to *PRDM1* in response to IL-21, in
441 nonfunctional CREBBP FLs treated by vorinostat, is likely not due to the decrease of
442 *BCL6* gene expression. We suspect that additional modifications on the BCL6 protein
443 complex could have been induced by the vorinostat allowing the decrease of BCL6
444 enrichment at INT3 and thereby suppressing the repression on *PRDM1*.

445 The loss of *PRDM1* contributes to the lymphomagenesis by blocking PC
446 differentiation (39), knowing however that in FLs the loss of CREBBP is not sufficient
447 to drive lymphomagenesis but need the co-occurrence of *BCL2* translocation (11).
448 Our analysis of four massively pretreated FL patients with a new pan-HDACi showed
449 that the drug might increase PC-identity genes expression within the tumor including
450 *PRDM1* in agreement with our *in vitro* data. The restoration of PC-identity genes with
451 a pan-HDACi is in line with the recent report of Jiang *et al.* who described broad
452 effects of CREBBP on the transcriptional regulation of B cells (19) and here, in our
453 study, specifically on a master gene of the B cell differentiation, *i.e.* *PRDM1*. Pan-
454 HDAC inhibitors may allow a new step in FL cell differentiation with varying efficacy
455 depending on the presence of somatic abnormalities and clonal tumor diversity. Re-
456 biopsied tissues presented an increase of the *BCL6* expression (~~data not shown~~),
457 which could be due to the re-progression status of the disease connected with the
458 proliferation properties of *BCL6* rather than its induction by the drug. Despite this
459 effect, the drug allowed a positive increase in the *PRDM1/BCL6* balance. Among the
460 identified up-regulated PC-identity genes we found *XBP1* gene, which occupies a
461 downstream position in the transcriptional cascade that governs B cell differentiation
462 (26). Indeed, *PRDM1/BLIMP-1* is known to regulate UPR components like *ATF6* and
463 *ERN1* that are required for full-length *XBP1* expression and the production of
464 subsequent active spliced form *XBP1s* (48,49). Two patients out of 3 increased
465 *XBP1s* and *ERN1* expressions with the pan-HDACi drug supporting the idea of a
466 successful *BLIMP1/PRDM1* protein restoration in these cells. Recently, Bujisic *et al.*
467 described that impaired *ERN1* expression and *XBP1* activation, contribute to tumor
468 growth in GC-type diffuse large B-cell lymphoma (50).

469 HDACi drugs seem to be promising medications in largely pretreated FLs as
470 confirmed recently by Even *et al.* who showed a significant clinical activity in this
471 disease, including highly durable responses (51). In this context our study shows that
472 monitoring effects of such drug by analysing specific gene expressions, *e.g.* *PRDM1*,
473 *IRF4*, *XBP1* and *ERN1* - thanks to iterative fine needle punctures to easily accessible
474 tumors - might be of interest. Furthermore, additional studies will also be needed to
475 confirm that pan-HDAC inhibitors restore both gene and protein expression for
476 *PRDM1*/Blimp1 and other key players in PC differentiation, thus providing the
477 ultimate proof of the effect of these drugs in FL. Collectively, our data uncover a new
478 mechanism by which pan-HDAC inhibitors may act positively to treat FL patients and
479 in particular those with nonfunctional CREBBP.

480

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22

493 French legal guidelines and fulfilled the requirements of the local institutional ethics
494 committee.

495

496 **Author Contribution**

497 F.D., M.R. and T.F. designed research; F.D., M.R., C.P., F.L-G., E.G., G.C., J.L.P
498 and C.H. performed research; F.D. and T.F. analyzed data and wrote the paper; V.
499 C-C, F.J. and M-A.B-R. assisted with experiments; P.G., V.R. and T.L. provided FL
500 samples; Resources and reading K.T.; Supervision and group leader T.F.

501 **Conflict of Interest**

502 The authors declare no competing interests

503

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- 689
690

691 **Figures Legends**

692 **Figure 1: Gene expression profiling revealed a functional impairment of the**
693 ***PRDM1*/Blimp1 response in FLs compared to normal centrocytes. (A)** Freshly
694 sorted normal CBs were cultured for 3h with or without CD40L+IL-21. Extracted
695 RNAs were subsequently hybridized on Affymetrix U133+2.0 microarrays and
696 expression of 10.000 probesets (PS) with the highest intensity were clustered using
697 unsupervised hierarchical clustering (HCA). Differentially expressed genes were
698 obtained for a FDR<0.05 comparing stimulated vs. unstimulated conditions (n=6). **(B)**
699 A new hierarchical clustering was performed only on a subset of genes known to be
700 up-regulated by either IL-21 or CD40L pathways or during the plasma cells
701 differentiation process. *P*-values (FDR) are calculated by moderated paired t-tests.
702 **(C)** CB and CC were sorted from tonsils based on the following markers

703 CD19^{pos}IgD^{neg}CD38^{hi}CD10^{pos} plus CXCR4-positive for CB only. Extracted RNAs
 704 were subsequently hybridized on Affymetrix U133+2.0 microarrays and same
 705 clustering strategy than in (A) was performed. List of genes corresponding to genes
 706 differentially expressed firstly in CB upon CD40L+IL-21 treatment and secondly
 707 between CB and CC were compared using Venn diagrams, thus identifying 804 up-
 708 and 965-down-regulated genes during CB/CC transition that could be linked to the
 709 CD40L+IL-21 stimulation. (D) FL B cells (n=10) were sorted based on
 710 CD20^{hi}CD44^{lo}CD38^{pos}IgD^{neg}CD138^{neg} definition. HCA was performed comparing CBs,
 711 CCs and FL B-cells for the 1,769 genes of the IL-21/CD40L-signature (corresponding
 712 to 804 up- and 965-down-regulated genes previously identified). Two PSs
 713 corresponding to the *PRDM1* gene are highlighted, and on right of the figure are
 714 indicated genes described further in the Panel F. (E) table shows proportions of
 715 genes highly or weakly expressed in CB, CC and FL for each box. (F) Ingenuity
 716 pathway enrichment on seven different items in Box1 compared to Box2. (G) *PRDM1*
 717 gene expression (for Affymetrix PS 228964_at) on a 23-independent cohort of FLs
 718 (FL-B) compared to reactive lymph nodes (RLN-B). In this study (14), B cells were
 719 obtained by immunomagnetic sorting based on the CD19 expression for both, FLs
 720 and normal tissues.

721

722 **Figure 2: *PRDM1* is not induced by IL-21 in FL B cells.**

723 *PRDM1* (A) and *BCL6* (B) expression in control L3055 (5 independent experiments)
 724 and in primary culture of purified FL B cells (n=18) stimulated with IL-21 for 24h and
 725 compared to the unstimulated condition (Unstim). Relative's quantities of *PRDM1*
 726 and *BCL6* genes were calculated after normalization to internal control gene

727 expressions and relative to an external calibrator used as control sample. Upon IL-21,
728 *PRDM1* expression was significantly induced in control L3055 whereas no
729 modification was observed in FL. For the *BCL6* expression no changes were
730 observed in control GBs L3055 neither in FL. Statistical analysis using Mann Whitney
731 test comparing globally all FLs upon IL-21 stimulation vs. unstimulated conditions
732 (see Supplementary Figure S3 C-D), were not significant. (C) *PRDM1* and *BCL6*
733 expressions upon IL-21 stimulation are correlated and the majority of FLs, mutated
734 for CREBBP, clustered in the lowers values of expression.

735

736 **Figure 3: Investigations of the IL-21/p-STAT3 signaling in FLs.** (A) Percentage of
737 Tfh from 15 FLs, 9 reactive lymph nodes (rLNs) and 7 tonsils; (B) Percentage of IL-
738 21 expressing Tfh from 5 FLs and 6 tonsils among the total Tfh population; (C) RMFI
739 comparison for pSTAT3 in Tfh from 15 FLs, 9 rLNs and 7 tonsils. (D) RMFI
740 comparison for pSTAT3 in 20 FL B-cells (BCL2-positive for tumor B cells and BCL2-
741 negative for nonmalignant B cells) and GC B-cell counterparts from 9 rLNs and 9
742 tonsils. (E) pSTAT3 RMFI correlation between BCL2-positive tumor B cells and
743 BCL2-negative nonmalignant B cells. (F) The relative expression of the pSTAT3
744 target gene *BATF* was increased after IL-21 stimulation in all FLs that we tested
745 (n=14). (G) Representative single or double immunohistochemistry staining for PD1
746 and pSTAT3 onto paraffin embedded tissues from one rLN (Left Panels) and a
747 representative case of FL (case FL_6103; Right Panels). In the rLN, PD1-positive
748 cells are polarized at the light zone of the GC and are associated with few pSTAT3-
749 positive cells. In FLs, PD1-positive cells are more frequent showing a diffuse
750 distribution and are associated with clusters of pSTAT3-positive cells. Unlike rLNs, in

751 FLs we visualized cells stained for both, PD1 and pSTAT3 (stars) corresponding to
752 Tfh. Significant observations are indicated as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

753

754 **Figure 4: CREBBP somatic mutations in FL and functional impact on PRDM1**
755 **response under IL-21 stimulation. (A)** Schematic representation (based on Human
756 Protein Database) of CREBBP and its key functional domains delimited with amino
757 acid positions with distribution of frameshift (green diamonds) and missense
758 mutations (brown diamonds) in 14 CREBBP-mutated FLs. Conserved domains are:
759 ZF, Zinc Finger-TAZ type; KIX, coactivator CBP (CREB-Binding protein and MYB
760 interaction domain); BD, Bromodomain; HAT, Histone Acetyl Transferase domain; ZZ,
761 Zinc Finger, ZZ type; NR: Nuclear receptor. **(B)** Besides control L3055, 10 FLs were
762 analyzed and classified in the table according to their HAT-CREBBP mutated status
763 (4 wild-type cases, plus control L3055, labeled WT; and 6 HAT-CREBBP mutated
764 cases). After 24h of culture with or without IL-21 (100ng/ μ L), cells were split for
765 subsequent RNA extraction or chromatin immunoprecipitation. Log₂ values of
766 IL21/Unstim fold change ratio (FC) of *PRDM1* expression from the Figure 2C are
767 indicated in this table with paralleled paired-FLs levels of BCL6 enrichment
768 IL21/Unstim ratio (Log₂) at the INT3 site of *PRDM1* relative to control IgG enrichment
769 used as control for each condition. In this table are also indicated the presence (+),
770 absence (-) or undetermined (nd) expression of BCL6 by immunohistochemistry
771 (BCL6 prot. IHC column) in parallel to the BCL6 translocation with t(3q27)
772 determined by FISH (3q27 pos. column).

773

774 **Figure 5: Assessment of BCL6 protein occupancy at the INT3 of PRDM1 in FL B**
775 **cells using the histone acetyl transferase inhibitor SAHA and impact on the**
776 **PRDM1/BCL6 expression balance.** Sorted FL B cells from 6 HAT/CREBBP-
777 mutated patients and control L3055 were cultured in presence of IL-21 with or without
778 addition of SAHA, or with SAHA alone. For each FL, fold-change ratio (FC) of the
779 tested condition compared to unstimulated condition (Unstim) was calculated for
780 PRDM1 (A) and BCL6 (B) expression, and for BCL6 enrichment (C). (D) The
781 PRDM1/BCL6 FC ratio was calculated comparing IL-21 alone (blue) to either,
782 SAHA+IL-21 (red) or SAHA alone (green). A significant statistical increase of
783 PRDM1 relative to BCL6 was observed for the former condition but not for the latter
784 one. L3055 data are shown, yellow triangle, for comparison. (E) Globally the addition
785 of SAHA to IL-21 compared to IL-21 alone induced an increase of PRDM1
786 expression concomitant with a decrease of BCL6 expression and a decrease of
787 BCL6 recruitment at INT3 of PRDM1 modifying the BCL6-PRDM1 balance.

788

789 **Figure 6: Mutation profiling and B cell differentiation gene expression analysis**
790 **of 4 FL patients treated with a new HDACi.** Patients with a refractory or multiple
791 relapsing FL were enrolled in a Phase 1 study (21) testing a new pan-HDACi inhibitor.
792 Variable response to the drug were observed for the four patients analyzed and
793 indicated as follow: NR, non-responder; SD, stable disease, CR: complete response
794 and OR: objective response. Samples were collected at diagnosis (Diag.) or after
795 drug administration (Drug.T.), except for the patient who reached a CR. Molecular
796 analyses were performed before and after treatment. (A) R-graphic representation
797 using “ggpubr” package, of clonal evolution following pan-HDACi administration

798 compared to diagnosis respective to variant allelic frequency (VAF) (VAF Diag. vs.
799 VAF Drug T.) of major variants found. **(B-C)** Results from RQ-PCRs analysis of basal
800 expression at the diagnosis and after treatment for *PRDM1*, *XBP1* and *IRF4* (**B**, in
801 green), for B cell-identity genes *BCL6*, *BACH2* and *PAX5* (**B**, in orange) and for
802 *XBP1s* and *ERN1* genes (**C**) relative to an external calibrator and normalized with
803 control gene expressions. **(D)** The expression ratio of each gene was calculated
804 comparing levels of expression after drug treatment to those at diagnosis. Plasma
805 cell-identity genes ratio are positive compared to B cell-identity genes, except for
806 *BCL6*.

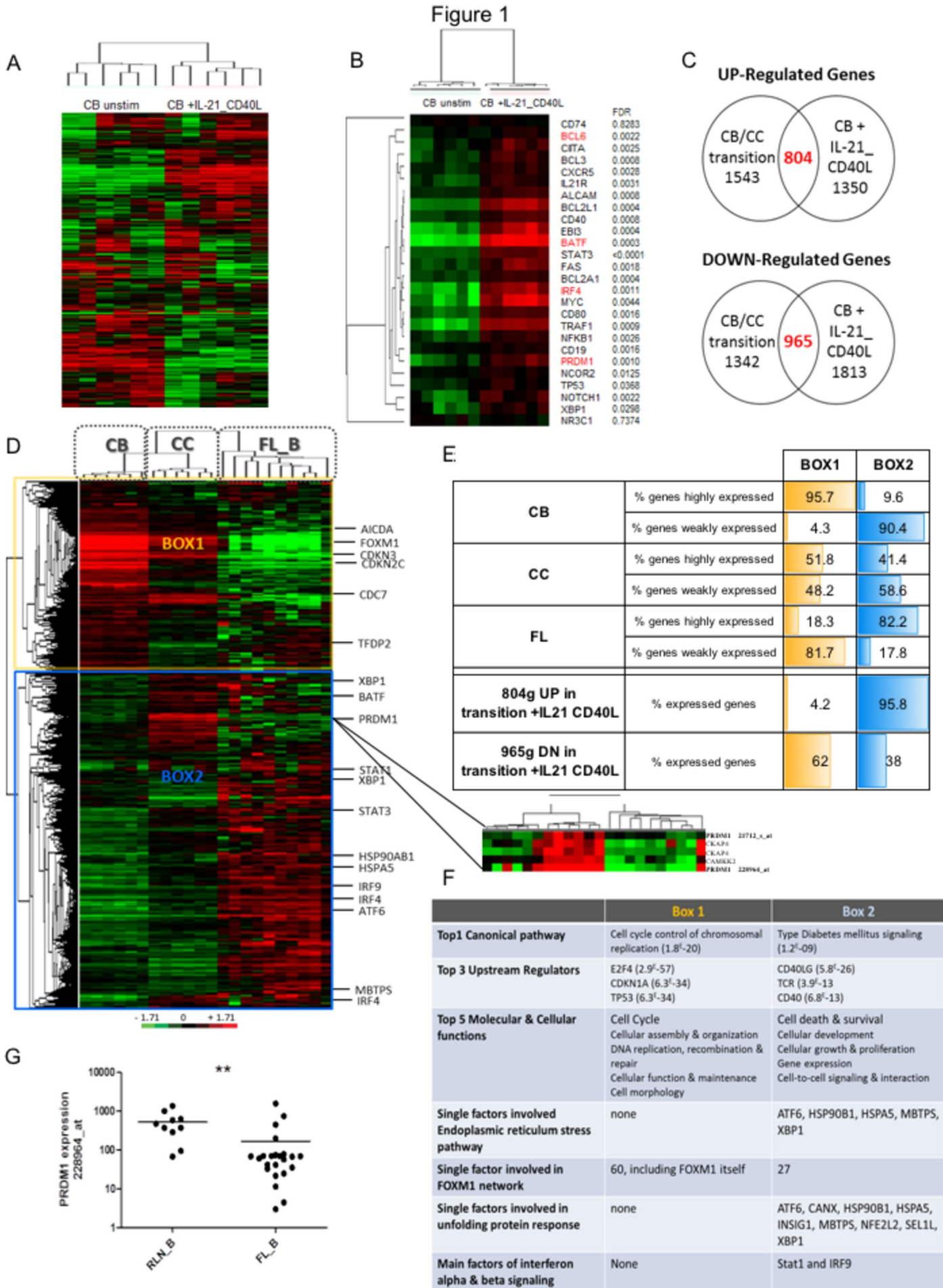


Figure 2

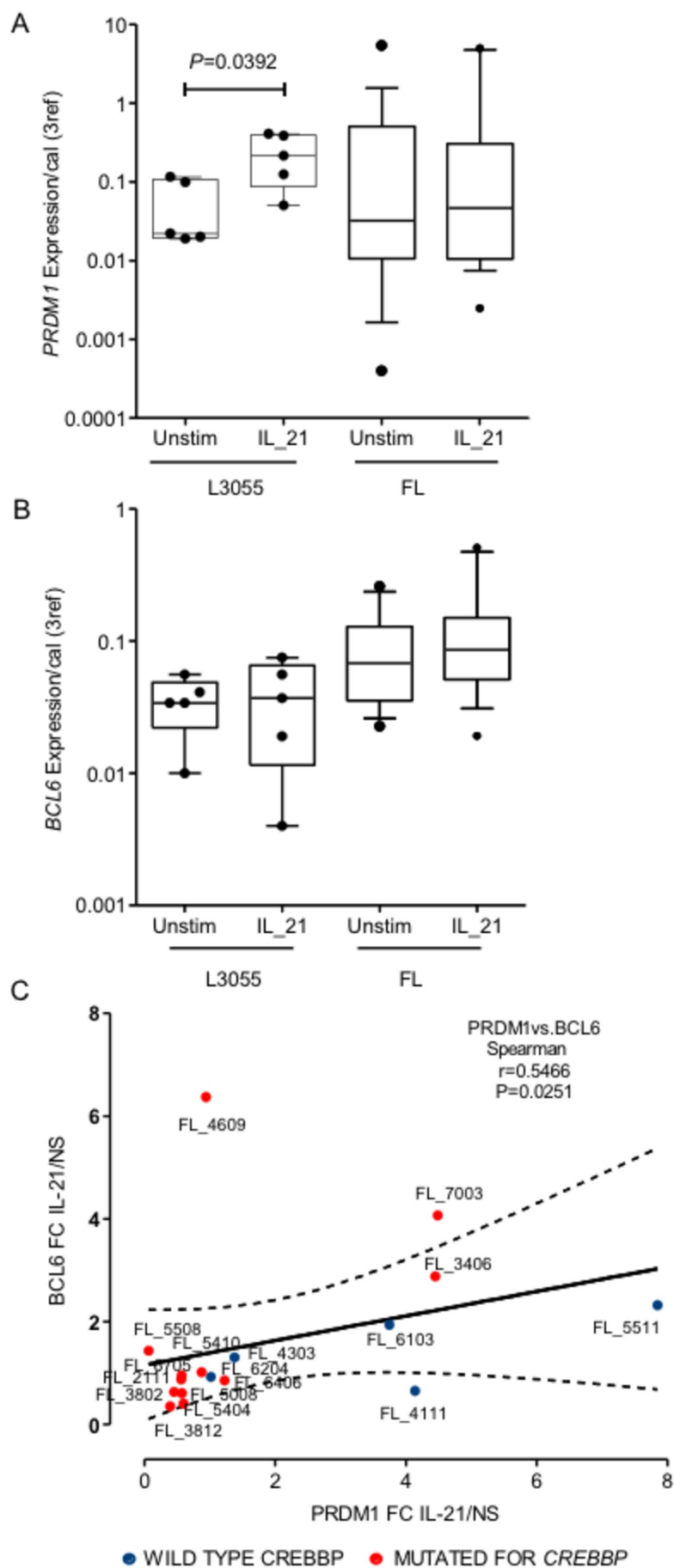


Figure 3

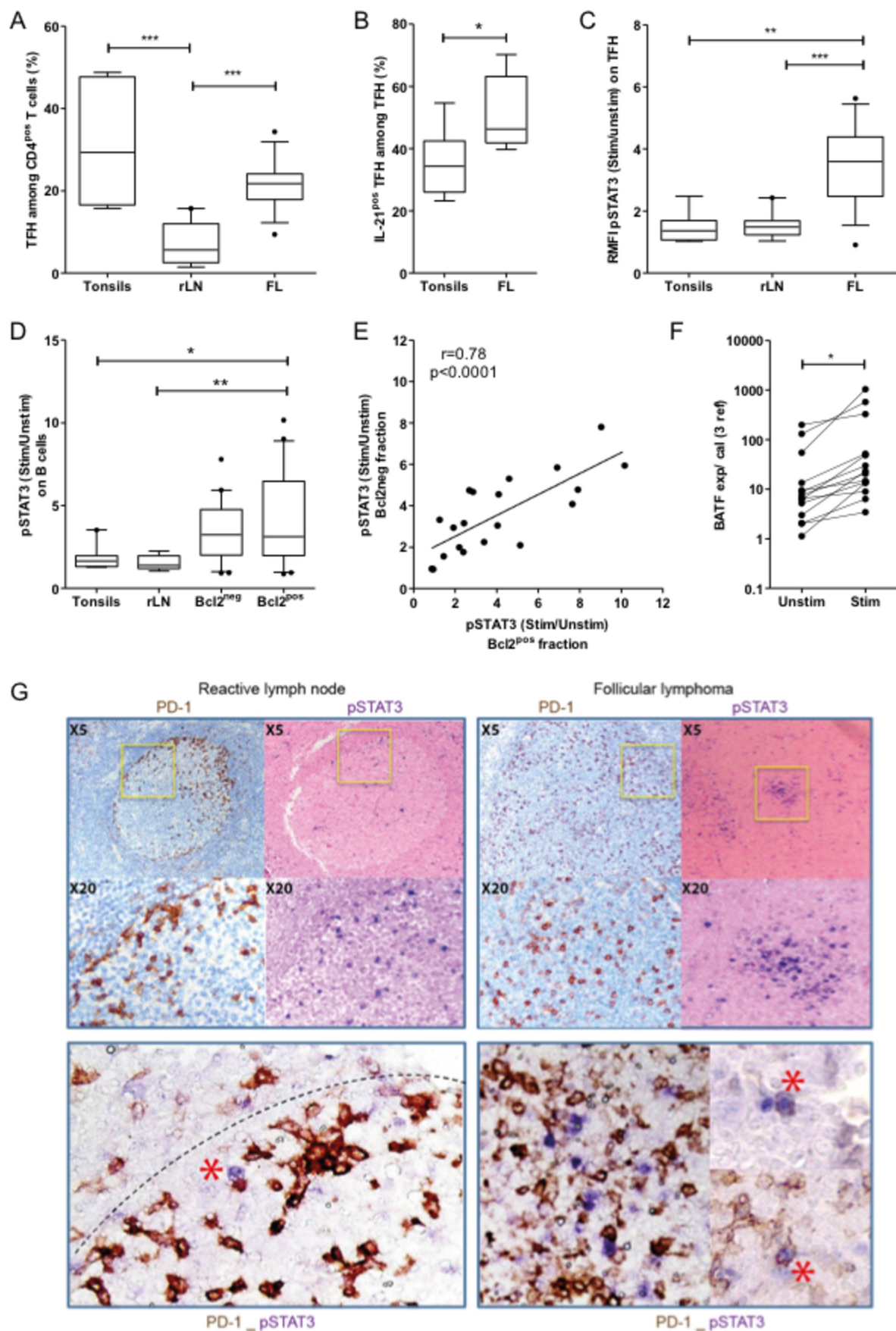
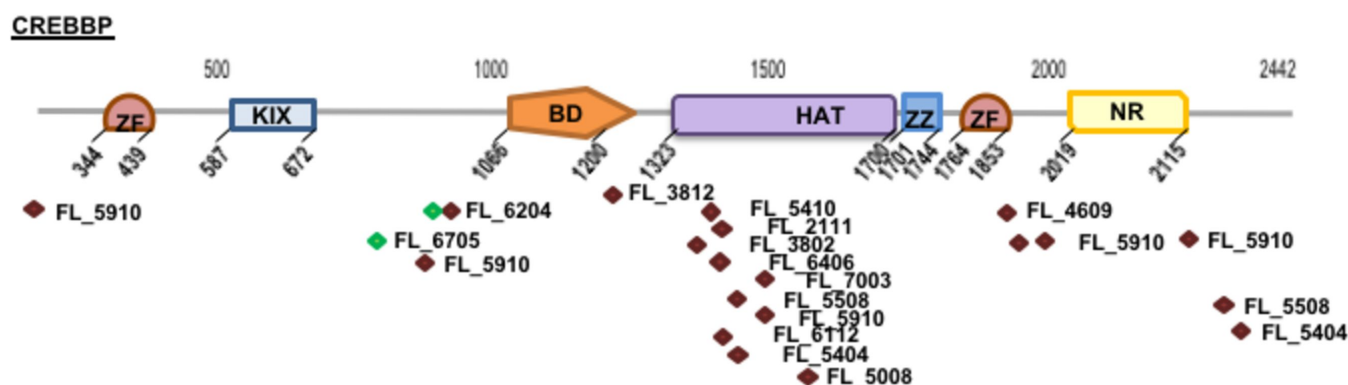


Figure 4

A



B

CREBBP HAT status	Cases	BCL6 prot. IHC	3q27 pos.	Log2 Exp. of <i>PRDM1</i> (FC IL21 / Unstim)	BCL6 Enrichment INT3 (Log2 ratio IL21 / Unstim)
WT	L3055	+	-	2.92	-3.22
	FL_6108	nd	-	0.57	-2.10
	FL_4303	nd	+	0.48	0.99
	FL_3406	+	+	1.63	2.08
	FL_5511	nd	+	0.41	-1.25
MUTATED	FL_6705 (E970fs)	nd	-	-0.82	1.67
	FL_5008 (L1621R)	+	-	-0.81	-0.07
	FL_7003 (Y1503D)	+	-	-0.21	2.86
	FL_5508 (Y1482H)	+	-	-3.99	4.45
	FL_5410 (D1435E)	+	-	-0.2	6.17
	FL_5404 (Y1482H)	+	-	-0.74	6.53

Exp. *PRDM1* WT vs MUT.; $P=0.0224$

BCL6 Enrich. WT vs MUT.; $P=0.0377$

Figure 5

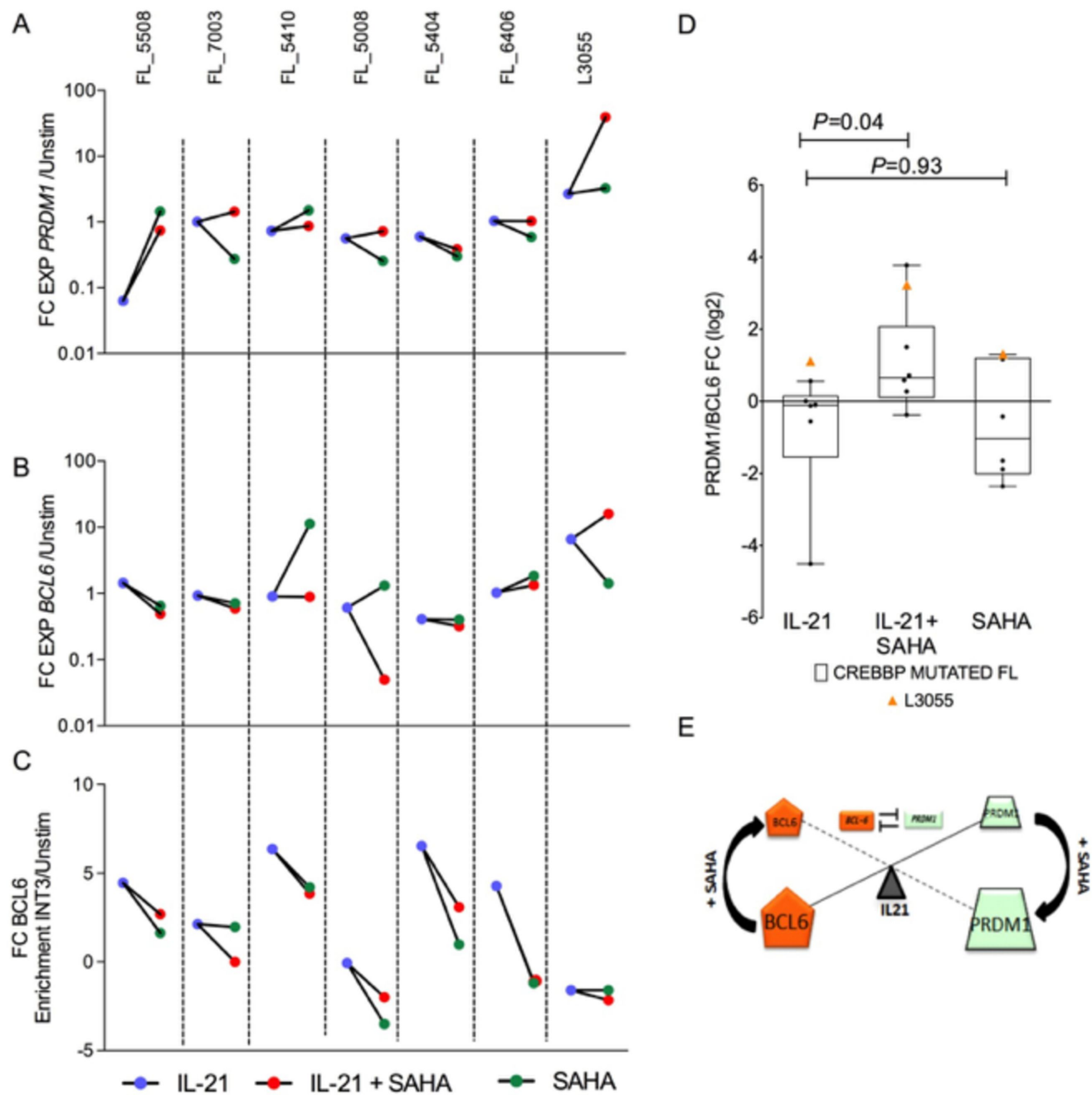


Figure 6

