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Running title: HDACi & *PRDM1* expression in FL

Keywords: Follicular lymphoma, *PRDM1*/BLIMP1, BCL6, HDACi, CREBBP

27 **RESEARCH MANUSCRIPT**

28 *Manuscript length* (excluding text of legends, tables and references): 4668 words

29 *Abstract length:* 222 words

30 *Translational Relevance:* 133 words

31 **NUMBER OF FIGURES:** 6

32 **REFERENCE COUNT:** 51

33 **PRESENCE OF SUPPLEMENTAL FIGURES & TABLES**

34

Translational Relevance

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

Abstract

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

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

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

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

Introduction

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

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

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

Material and Methods

Samples

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

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

Transcriptomic data

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

Gene expression analysis

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

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

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

Culture conditions and molecular analyses

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

Somatic mutations assessment

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

Statistical analyses

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

Results

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

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

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

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

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

Impaired PRDM1 response after IL-21 stimulation in FL

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

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

FL Tfhs and FL B cells show a functional and increased IL-21/pSTAT3 response

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

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

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

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

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

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

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

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

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

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

Our investigations on tumor RNA extracts showed globally an increase in the expression of the 3 tested PC transcription factors, *PRDM*1, *IRF4* and *XBP1* in re-biopsies compared to the initial tissues while B cell-identity factors remain stable except for BCL6, and BACH2 in patient FL_CON only (Figure 6B). The expression of *PRDM1* in FL_CON and FL_CAS increased during treatment whereas FL_DAD showed stable expression but had a higher level of *PRDM1* expression at the time of inclusion compared to other patients. In addition, FL_DAD was the only one who showed a marked increase in the expression of *IRF4*. The 3 patients for whom the treatment failed showed a clear increase of *XBP1* expression. Interestingly, for patients FL_DAD and FL_CON, we observed an elevation of the expression of the active form of *XBP1*, *XBP1s,* associated with a clear up-regulation of the UPR (unfolding protein response) sensor *ERN1*, a gene encoding the endoplasmic reticulum kinase involved in XBP1 maturation (Figure 6C) (35). These mechanisms are essential for PC differentiation (36). Overall, increased expressions of key PC-identity genes under pan-HDACi treatment suggest that FLs underwent some differentiation but that tumor cells still escape the drug (Figure 5D).

Discussion

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

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

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

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

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

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

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

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

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

Acknowledgments

This work was supported by an internal grant from the Hematology laboratory, CHU de Rennes, France, and the Ligue Régionale contre le Cancer de l'Ouest. The NGS experiments are part of the RELYSE project supported by a National Cancer Institute translational grant. Sequencing were performed in the Biogenouest Genomics / Human & Environmental Genomics core facility of Rennes (Biosit/OSUR). Human samples were obtained from the processing of biological samples through the Centre de Ressources Biologiques (CRB)-Santé of Rennes (BB-0033-00056, http://www.crbsante-rennes.com). Cell sorting was performed by Flow Cytometry facility, Biosit, University of Rennes 1. Part of this work was supported by the Carte d'identité des Tumeurs (CIT) program (http://cit.ligue-cancer.net/index.php/en) from the Ligue Nationale Contre le Cancer. The research protocol was conducted under

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

Author Contribution

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

Conflict of Interest

The authors declare no competing interests

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Figures Legends

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

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

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

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

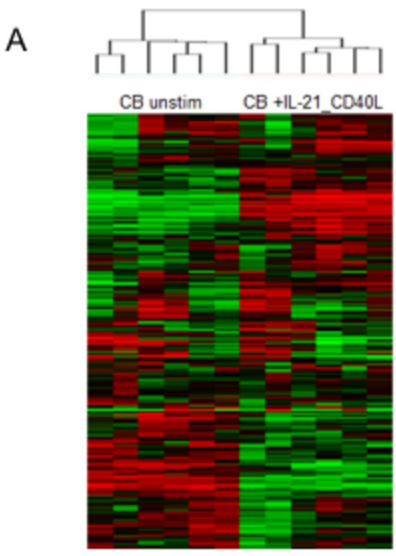
Figure 4: *CREBBP* somatic mutations in FL and functional impact on *PRDM1* response under IL-21 stimulation. (**A**) Schematic representation (based on Human Protein Database) of CREBBP and its key functional domains delimited with amino acid positions with distribution of frameshift (green diamonds)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

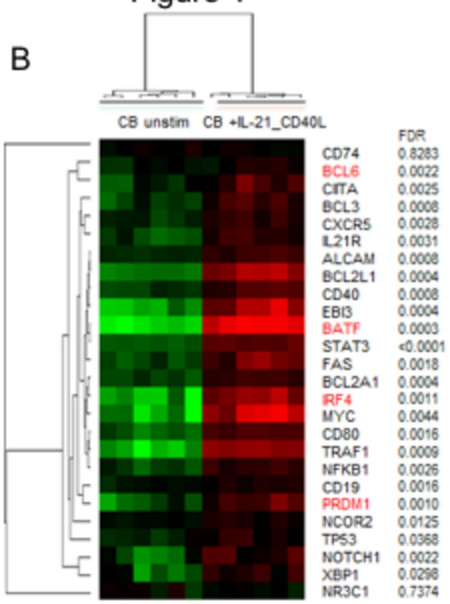
Figure 5: **Assessment of BCL6 protein occupancy at the INT3 of *PRDM1* in FL B cells using the histone acetyl transferase inhibitor SAHA and impact on the PRDM1/BCL6 expression balance**. Sorted FL B cells from 6 HAT/CREBBP-mutated patients and control L3055 were cultured in presence of IL-21 with or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

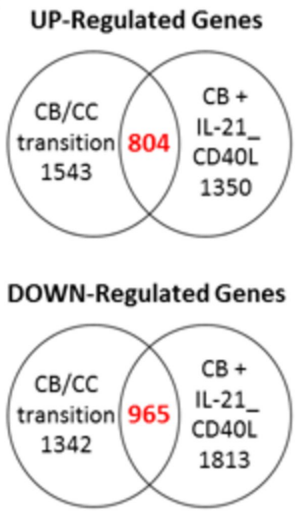
Figure 6: **Mutation profiling and B cell differentiation gene expression analysis of 4 FL patients treated with a new HDACi**. Patients with a refractory or multiple relapsing FL were enrolled in a Phase 1 study (21) testing a new pan-HDACi inhibitor. Variable response to the drug were observed for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

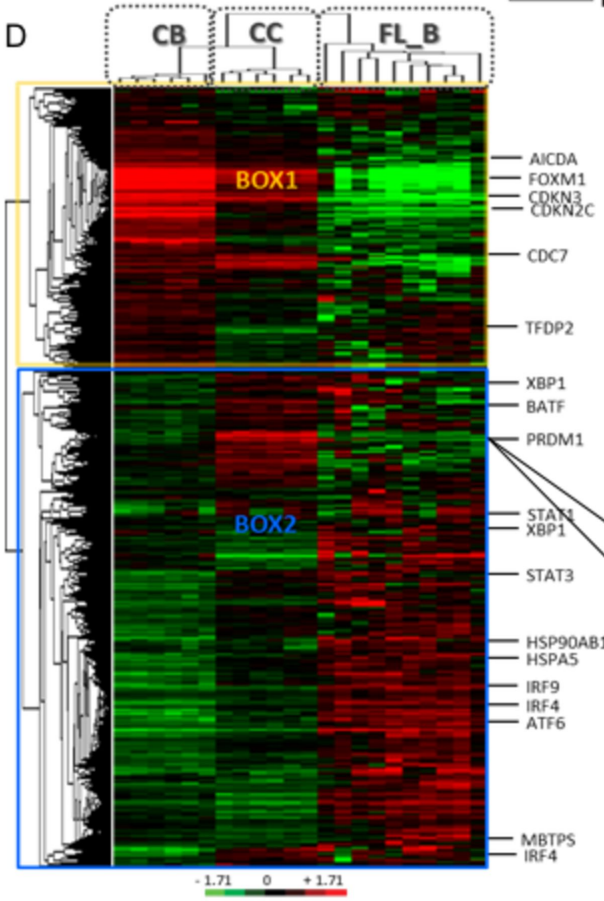
compared to diagnosis respective to variant allelic frequency (VAF) (VAF Diag. *vs.* VAF Drug T.) of major variants found. (**B-C**) Results from RQ-PCRs analysis of basal expression at the diagnosis and after treatment for *PRDM1, XBP1 and IRF4* (**B**, in green), for B cell-identity genes *BCL6, BACH2*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

Figure 1



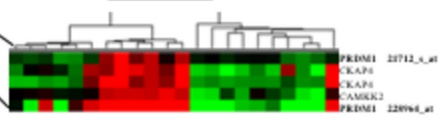






E

		BOX1	BOX2
CB	% genes highly expressed	95.7	9.6
	% genes weakly expressed	4.3	90.4
CC	% genes highly expressed	51.8	41.4
	% genes weakly expressed	48.2	58.6
FL	% genes highly expressed	18.3	82.2
	% genes weakly expressed	81.7	17.8
804g UP in transition +IL21 CD40L	% expressed genes	4.2	95.8
965g DN in transition +IL21 CD40L	% expressed genes	62	38



F

	Box 1	Box 2
Top1 Canonical pathway	Cell cycle control of chromosomal replication (1.8^{E}-20)	Type Diabetes mellitus signaling (1.2^{E}-09)
Top 3 Upstream Regulators	E2F4 (2.9^{E}-57) CDKN1A (6.3^{E}-34) TP53 (6.3^{E}-34)	CD40LG (5.8^{E}-26) TCR (3.9^{E}-13 CD40 (6.8^{E}-13)
Top 5 Molecular & Cellular functions	Cell Cycle Cellular assembly & organization DNA replication, recombination & repair Cellular function & maintenance Cell morphology	Cell death & survival Cellular development Cellular growth & proliferation Gene expression Cell-to-cell signaling & interaction
Single factors involved Endoplasmic reticulum stress pathway	none	ATF6, HSP90B1, HSPA5, MBTPS, XBP1
Single factor involved in FOXM1 network	60, including FOXM1 itself	27
Single factors involved in unfolding protein response	none	ATF6, CANX, HSP90B1, HSPA5, INSIG1, MBTPS, NFE2L2, SEL1L, XBP1
Main factors of interferon alpha & beta signaling	None	Stat1 and IRF9

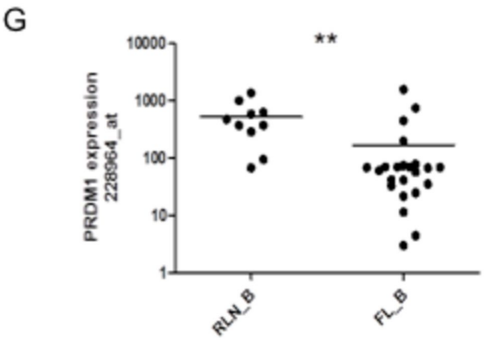


Figure 2

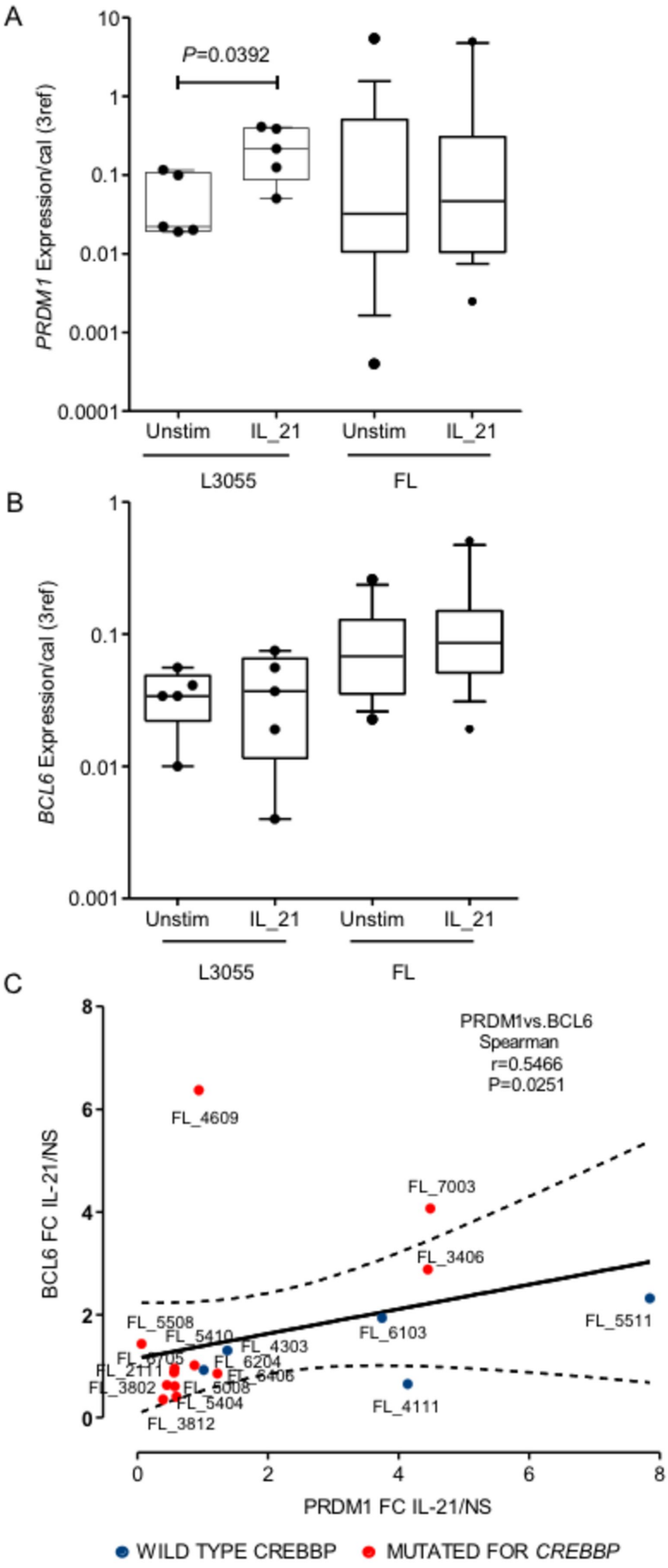
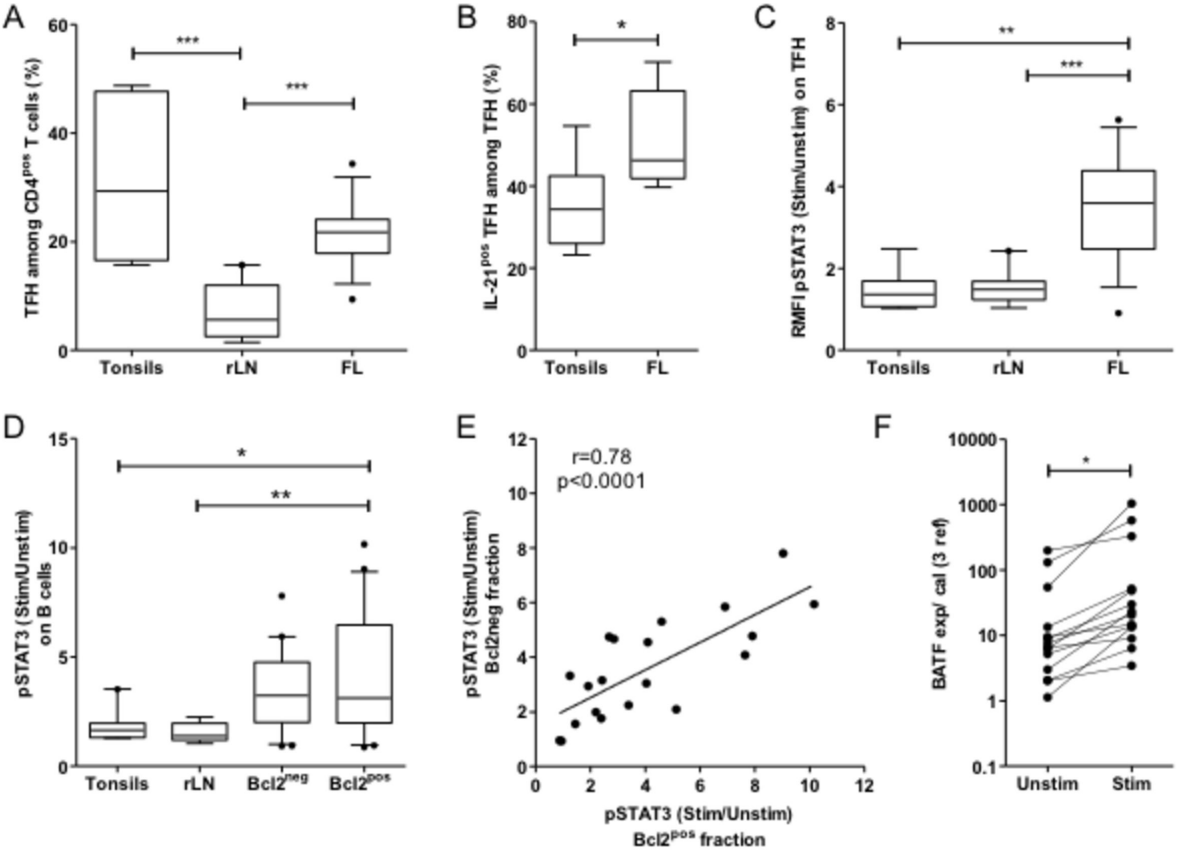

A
P=0.0392
PRDM1 Expression/cal (3ref)
10
1
0.1
0.01
0.001
0.0001
Unstim
IL_21
Unstim
IL_21
L3055
FL
B
BCL6 Expression/cal (3ref)
1
0.1
0.01
0.001
Unstim
IL_21
Unstim
IL_21
L3055
FL
C
PRDM1vs.BCL6
Spearman
r=0.5466
P=0.0251
BCL6 FC IL-21/NS
PRDM1 FC IL-21/NS
FL_4609
FL_7003
FL_3406
FL_5511
FL_6103
FL_4303
FL_5508
FL_5410
FL_6204
FL_2111
FL_3802
FL_5008
FL_5404
FL_3812
FL_4111
WILD TYPE CREBBP
MUTATED FOR CREBBP

Figure 3


A
TFH among CD4pos T cells (%)
***
***
Tonsils
rLN
FL
B
IL-21pos TFH among TFH (%)
*
Tonsils
FL
C
RMFI pSTAT3 (Stim/unstim) on TFH
**
***
Tonsils
rLN
FL
D
pSTAT3 (Stim/Unstim) on B cells
*
**
Tonsils
rLN
Bcl2neg
Bcl2pos
E
r=0.78
p<0.0001
pSTAT3 (Stim/Unstim) Bcl2neg fraction
pSTAT3 (Stim/Unstim) Bcl2pos fraction
F
BATF expl cal (3 ref)
*
Unstim
Stim

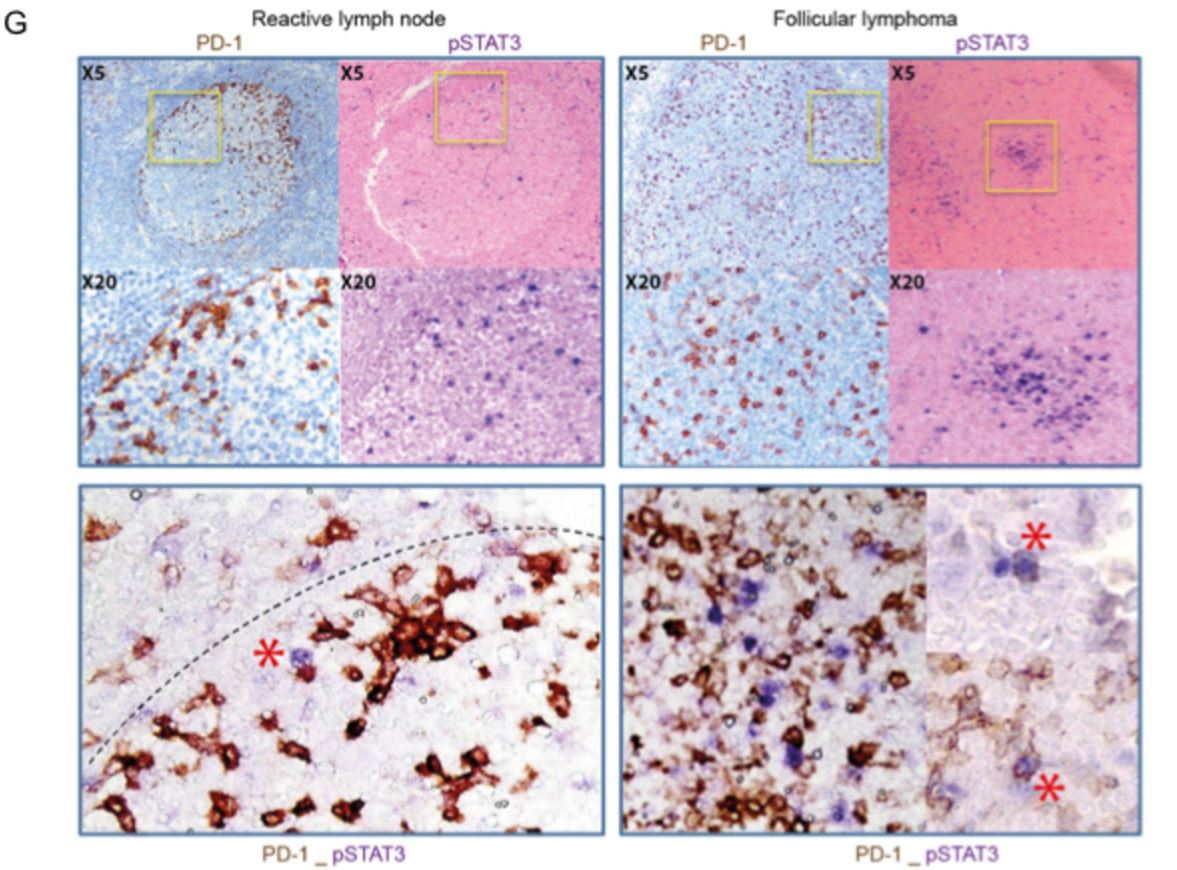
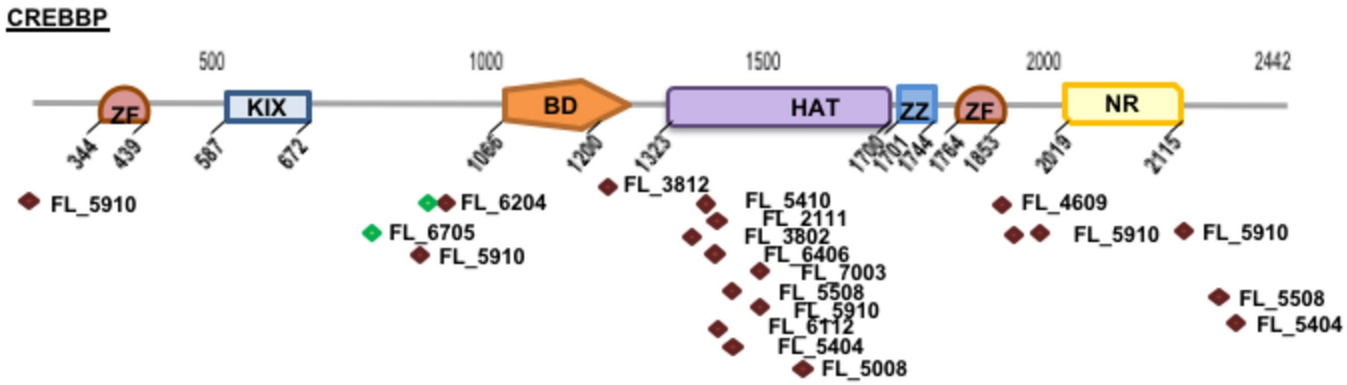

G
Reactive lymph node
Follicular lymphoma
PD-1
pSTAT3
X5
X20
PD-1 _ pSTAT3

Figure 4

A



B

CREBBP HAT status	Cases	BCL6 prot. IHC	3q27 pos.	Log2 Exp. of *PRDM1* (FC IL21 / Unstim)		BCL6 Enrichment INT3 (Log2 ratio IL21 / Unstim)	
WT	L3055	+	-	2.92		-3.22	
	FL_6108	nd	-	0.57	Exp. *PRDM1* WT vs MUT.; *P*=0.0224	-2.10	BCL6 Enrich. WT vs MUT.; *P*=0.0377
	FL_4303	nd	+	0.48		0.99	
	FL_3406	+	+	1.63		2.08	
	FL_5511	nd	+	0.41		-1.25	
MUTATED	FL_6705 (E970*fs*)	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	

Figure 5

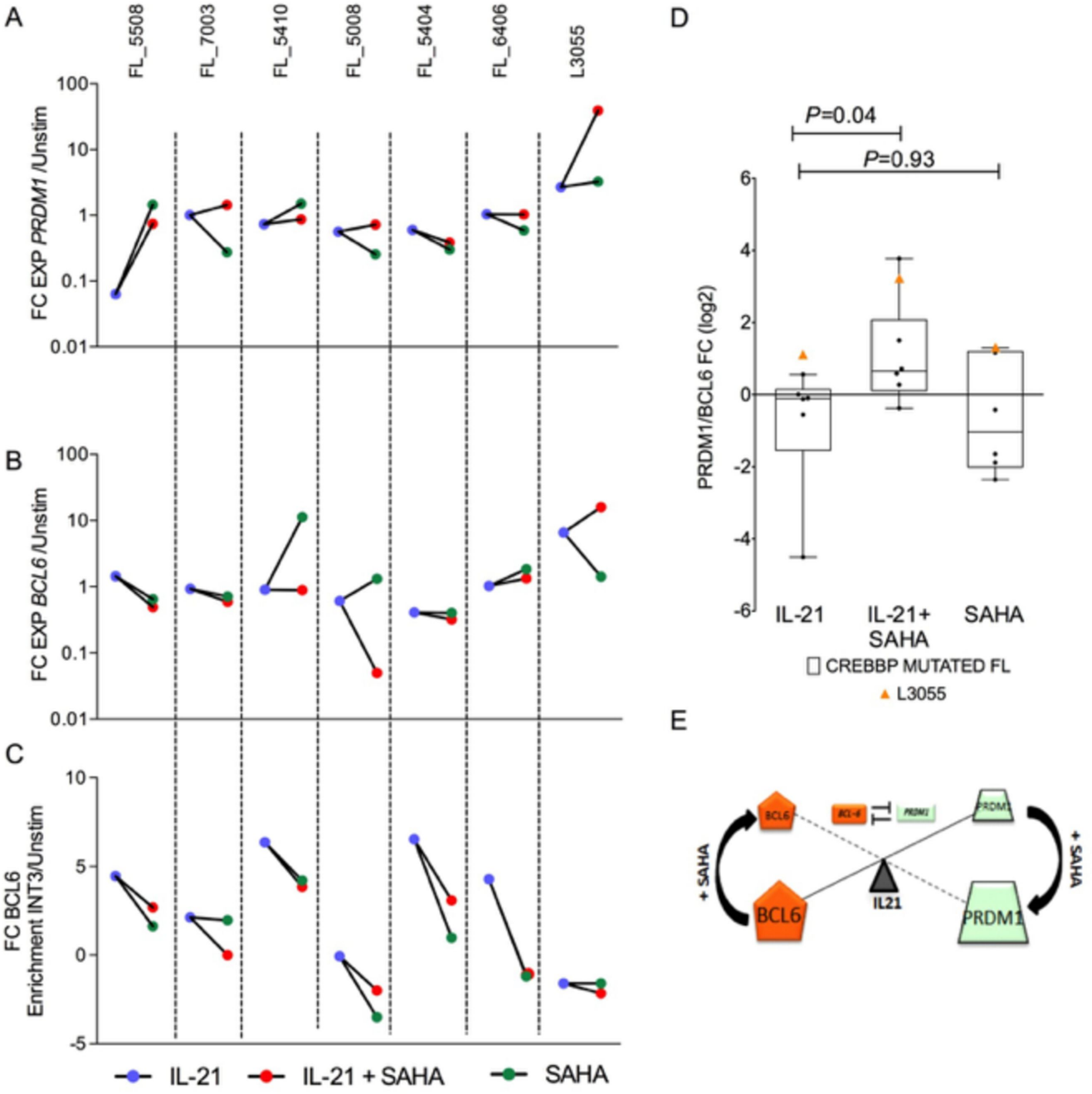
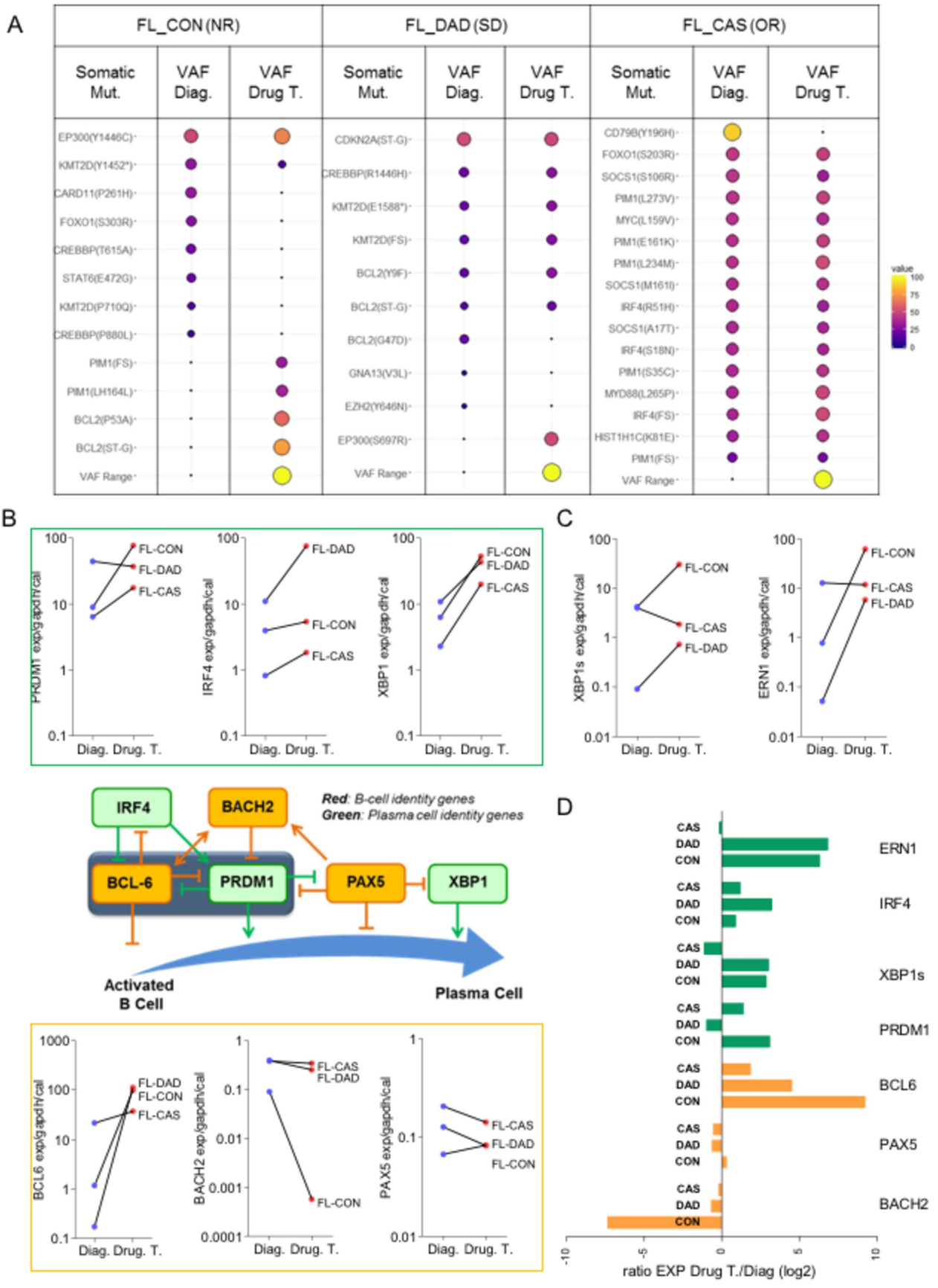

A
FL_5508
FL_7003
FL_5410
FL_5008
FL_5404
FL_6406
L3055
FC EXP PRDM1 /Unstim
100
10
1
0.1
0.01
B
FC EXP BCL6 /Unstim
100
10
1
0.1
0.01
C
FC BCL6
Enrichment INT3/Unstim
10
5
0
-5
IL-21
IL-21 + SAHA
SAHA
D
P=0.04
P=0.93
PRDM1/BCL6 FC (log2)
6
4
2
0
-2
-4
-6
IL-21
IL-21+ SAHA
SAHA
CREBBP MUTATED FL
L3055
E
BCL6
PRDM1
+ SAHA
+ SAHA
IL21
BCL6
PRDM1

Figure 6


A
FL_CON (NR)
FL_DAD (SD)
FL_CAS (OR)
Somatic Mut.
VAF Diag.
VAF Drug T.
EP300(Y1446C)
KMT2D(Y1452*)
CARD11(P261H)
FOXO1(S303R)
CREBBP(T615A)
STAT6(E472G)
KMT2D(P710Q)
CREBBP(P880L)
PIM1(FS)
PIM1(LH164L)
BCL2(P53A)
BCL2(ST-G)
VAF Range
CDKN2A(ST-G)
CREBBP(R1446H)
KMT2D(E1588*)
KMT2D(FS)
BCL2(Y9F)
BCL2(ST-G)
BCL2(G47D)
GNA13(V3L)
EZH2(Y646N)
EP300(S697R)
VAF Range
CD79B(Y196H)
FOXO1(S203R)
SOCS1(S106R)
PIM1(L273V)
MYC(L159V)
PIM1(E161K)
PIM1(L234M)
SOCS1(M161I)
IRF4(R51H)
SOCS1(A17T)
IRF4(S18N)
PIM1(S35C)
MYD88(L265P)
IRF4(FS)
HIST1H1C(K81E)
PIM1(FS)
VAF Range
value
100
75
50
25
0
B
PRDM1 exp/gapdh/cal
IRF4 exp/gapdh/cal
XBP1 exp/gapdh/cal
FL-CON
FL-DAD
FL-CAS
Diag. Drug. T.
C
XBP1s exp/gapdh/cal
ERN1 exp/gapdh/cal
FL-CON
FL-CAS
FL-DAD
Diag. Drug. T.
IRF4
BACH2
BCL-6
PRDM1
PAX5
XBP1
Red: B-cell identity genes
Green: Plasma cell identity genes
Activated B Cell
Plasma Cell
BCL6 exp/gapdh/cal
BACH2 exp/gapdh/cal
PAX5 exp/gapdh/cal
FL-DAD
FL-CON
FL-CAS
FL-CAS
FL-DAD
FL-CON
FL-CAS
FL-DAD
FL-CON
Diag. Drug. T.
D
CAS
DAD
CON
ERN1
IRF4
XBP1s
PRDM1
BCL6
PAX5
BACH2
ratio EXP Drug T./Diag (log2)