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A hybrid mechanism of action for BCL6 in B-cells defined by formation of functionally distinct complexes at enhancers and promoters

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SUMMARY

The BCL6 transcriptional repressor is required for development of germinal center (GC) B-cells and diffuse large B-cell lymphomas (DLBCL). Although BCL6 can recruit multiple corepressors, its transcriptional repression mechanism of action in normal and malignant B-cells is unknown. We find that in B-cells, BCL6 mostly functions through two independent mechanisms that are

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collectively essential to GC formation and DLBCL, both mediated through its N-terminal BTB domain. These are: i) formation of a unique ternary BCOR-SMRT complex at promoters with each corepressor binding to symmetrical sites on BCL6 homodimers, linked to specific epigenetic chromatin features, and ii) the "toggling" of active enhancers to a poised but not erased conformation through SMRT-dependent H3K27 de-acetylation, which is mediated by HDAC3 and opposed by p300 histone acetyltransferase. Dynamic toggling of enhancers provides a basis for B-cells to undergo rapid transcriptional and phenotypic changes in response to signaling or environmental cues.

INTRODUCTION

The BCL6 transcriptional repressor is required for formation of germinal centers (GC) during T-cell dependent immune responses (Ci et al., 2008). BCL6 also plays a critical role in initiation and maintenance of B-cell lymphomas derived from GC B-cells such as diffuse large B-cell lymphomas (DLBCL)(Ci et al., 2008). Defining the mechanism of action of BCL6 is of crucial importance to understanding the biology of B-cells and the molecular pathogenesis of BCL6-dependent lymphoid neoplasms. BCL6 is a member of the BTB-POZ – C2H2 zing finger family of transcription factors (Stogios et al., 2005). The BCL6 BTB domain has autonomous repressor activity and folds as an obligate homodimer (Ahmad et al., 2003). The dimer interface forms two extended grooves that serve as docking sites for three corepressors, SMRT, NCOR and BCOR (Ahmad et al., 2003; Ghetu et al., 2008). SMRT and NCOR are highly conserved and bind to the BCL6 BTB groove with an identical peptide sequence. They form a complex with TBL1, TBLR1, GPS2 and HDAC3, and allosterically enhance HDAC3-mediated H3K9 acetylation (Karagianni and Wong, 2007). BCOR shares no sequence or structure similarity with SMRT/NCOR and binds to BCL6 using a completely different peptide sequence (Ahmad et al., 2003; Ghetu et al., 2008). BCOR forms a Polycomb Repressor Complex 1 (PRC1)-like complex with PCGF1, KDM2B, RING1, SKP1, RYBP and RNF2 (Farcas et al., 2012; Gao et al., 2012; Gearhart et al., 2006; Sanchez et al., 2007). BTB point mutations that disrupt corepressor recruitment inactivate BTB domain repressor function (Ahmad et al., 2003; Ghetu et al., 2008). A similar effect can be achieved using specific BCL6 BTB groove binding peptides or small molecules (Cerchietti et al., 2010a; Cerchietti et al., 2009; Polo et al., 2004). The BTB domain corepressor interaction is an important mediator of BCL6 actions and a potential therapeutic target (Ci et al., 2008; Parekh et al., 2008). Yet it is not known how these protein interactions translate into transcriptional repression and where and how different BCL6 complexes assemble in the genome. Herein we confirm that BTB-corepressor interactions are absolutely required for survival of both malignant and normal B-cells. We show that BCL6 mediates these effects through two functionally distinct mechanisms. The first involves formation of a unique ternary complex whereby BCL6 can coordinate the actions of the BCOR Polycomb-like complex with SMRT/NCOR to potently repress target genes. The second involves a novel mechanism for "toggling" active enhancers into a "poised" configuration, through SMRT-HDAC3 dependent H3K27 deacetylation. This new function for HDAC3 enables BCL6-SMRT complexes to compete with p300 in switching enhancers between "on" and "off" states. Reversible enhancer toggling may be critical for dynamic modulation of the BCL6 transcriptional program during the GC reaction as well for the therapeutic effects of BCL6 inhibitors.

RESULTS

Distinct genomic localization patterns of specific BCL6-corepressor complexes

To evaluate the full impact of disrupting BCL6 BTB domain interactions with corepressors in DLBCL cells we treated mice bearing human DLBCL cell line xenografts with RI-BPI, a

peptidomimetic that specifically disrupts the BCL6 BTB domain interaction with SMRT, NCOR and BCOR corepressors (Cerchietti et al., 2009; Polo et al., 2004). Low doses of RI-BPI (25 mg/kg/d) given to mice were shown to slow DLBCL tumor growth (Cerchietti et al., 2009). In the current study we administered RI-BPI (50 mg/kg) or control peptide for 5 days to mice bearing established human DLBCL xenografts. RI-BPI caused complete regression of fully established DLBCL tumors in 100% of mice (Figure 1A). There was no microscopic evidence of residual tumor or tumor regrowth after treatment discontinuation in 60% of these mice. Hence the BCL6 BTB domain corepressor recruitment is essential for the survival of BCL6 dependent human DLBCL cells. To dissect out the transcriptional mechanisms through which BCL6 and its corepressors mediate these essential functions we next performed ChIP-seq for these proteins in DLBCL cells (OCI-Ly1). All ChIP-seq assays met ENCODE quality criteria (Table S1). Using stringent peak detection thresholds and the overlap of two highly correlated biological replicates ($r = 0.84$), we identified 14,780 BCL6 binding sites corresponding to the most highly enriched peaks (Figure S1A–B). Most BCL6 peaks localized to intronic (42%) and intergenic regions (31%), whereas 23% located to promoters (Figure 1B). The BCL6 DNA binding motif (Ci et al., 2009) was highly overrepresented ($p<1e-8$) and preferentially localized near the BCL6 peak summits (Figure S1C). BCL6 was enriched at well-known BCL6 targets such as *BCL6* itself (Wang et al., 2002), *PRDM1* (Shaffer et al., 2000), *TP53* (Phan and Dalla-Favera, 2004), *EP300* (Cerchietti et al., 2010b), *BCL2* (Ci et al., 2009; Saito et al., 2009) and *ATR* (Ranuncolo et al., 2007) (Figure S1D).

Our ChIP-seq analysis of BCL6 corepressors identified 4379 SMRT, 4302 NCOR and 17548 BCOR high quality peaks (Figure S1E–G). Strikingly 90% of SMRT and NCOR peaks overlapped with BCL6, suggesting that their function is mostly tied to BCL6 in DLBCL (Figure 1C and Figure S1G). Even though NCOR and SMRT can bind to many transcription factor partners (Perissi et al.) it appears that association with BCL6 is their dominant function in the B-cell context. Reciprocally only 27% of BCL6 peaks were occupied by NCOR-SMRT. BCL6-SMRT and BCL6-NCOR complexes exhibit extensive binding in intergenic and intronic regions with proportionally less promoter binding (Figure 1B). Because SMRT and NCOR were mostly colocalized and have similar biochemical functions ($r = 0.76$, Pearson, Figure S1E) we focused on SMRT for subsequent analyses. BCOR occupied 36% of BCL6 peaks and was more widely distributed to non-BCL6 containing peaks than SMRT/NCOR suggesting that it may have BCL6 independent functions (Figure 1C). In contrast to BCL6-SMRT, BCL6-BCOR complexes were more frequently localized to promoters (Figure 1B). Consistent with previous studies (Ci et al., 2009) BCL6 corepressor peaks contain binding sites for other transcription factors (including STAT sites (which overlap with BCL6 motif (Dent et al., 1997)) RUNX1 and ELK1), which might either compete or cooperate with BCL6. BCOR-BCL6 peaks were preferentially enriched in CG rich sequences, consistent their frequent localization in CpG-islands (35%; 1830/5265 peaks). On the other hand, BCL6-SMRT peaks were preferentially enriched in MEF2A motifs (Figure 1H). Notably, 13% of BCL6 binding sites contain both SMRT and BCOR peaks, suggesting that BCL6 may simultaneously recruit both corepressors at certain BCL6 binding sites (Figure 1C). We also performed ChIP-seq for BCL6, SMRT, NCOR and BCOR in purified primary human GC B-cells, from which DLBCLs arise (Figure S1I–J). Seventy eight percent of BCL6 target genes in DLBCL cells overlapped with GC B-cells, and 85% of target genes with BCL6-corepressor complexes in DLBCL also contained such complexes in GC B-cells, although GC B-cells also have additional unique targets (Figure S1K–L). Most importantly, the genome-wide distribution of BCL6 and corepressors were highly similar to DLBCL cells with comparable distributions to promoters and intergenic/intronic regions and ∼90% overlap of SMRT with BCL6 (Figure S1M–N). These results suggest that recruitment of these corepressors may be just as vital for normal GC B-cells as for DLBCL cells. Confirming this hypothesis, knockin

mice expressing a $BCL6^{N21KH116A}$ lateral groove mutant that is unable to recruit SMRT, NCOR and BCOR, but is otherwise normally expressed, folded and bound to target genes (Ahmad et al., 2003; Ghetu et al., 2008), fail to form GCs (Figure S1O)(Huang et al., 2013).

BCL6 forms SMRT/BCOR ternary complexes to potently repress expression

To understand the significance of BCL6 and corepressor distribution patterns relative to gene expression we initially focused on BCL6 promoter complexes. BCL6 was bound to the promoters of 3140 genes in DLBCL cells, 71% of which were occupied by overlapping BCL6-corepressor peaks. Overall, BCL6 binding sites at promoters could be classified into four classes: i) BCL6 only (n=906), ii) BCL6-SMRT (n=92), iii) BCL6-BCOR (n=1783) and iv) BCL6-SMRT-BCOR (n=341) (Figure S1P). At these latter sites BCL6-SMRT-BCOR were all colocalized suggesting that these are BCL6-dependent ternary complexes. The requirement of BCL6 to recruit BCOR and SMRT was confirmed by performing ChIP assays at representative promoters (*PRDM1*, *TLR4* and *CD69*) 24 h after BCL6 or control siRNA transduction in DLBCL cells. Recruitment of both corepressors was reduced proportionally to BCL6 depletion (Figure S1Q).

To determine the relative contribution of these different BCL6 complexes to gene expression we performed mRNA-seq at 24 h and 48 h after transduction of BCL6 or control siRNA in DLBCL cells (Figure S1R–S). Derepression of BCL6 promoter target genes was the dominant effect after BCL6 knockdown (approximately 70% of genes upregulated). We used gene set enrichment analysis (GSEA) to determine which type of BCL6 complex (BCL6 only, BCL6-BCOR, BCL6-SMRT, and BCL6-SMRT-BCOR) was most strongly associated with gene derepression (Figure 1D). This analysis revealed strong enrichment of BCL6 ternary complex (BCL6-SMRT-BCOR) among derepressed genes (FDR=0.002). BCL6-BCOR promoters were mildly enriched in derepressed genes with only a trend towards statistical significance (FDR=0.088). Genes bound by BCL6-SMRT only or BCL6 without corepressors were not significantly affected by BCL6 depletion (FDR=0.22 and FDR=0.99 respectively). Accordingly BCL6 ternary complex genes were more significantly derepressed when compared with BCL6 only, BCL6-SMRT or BCL6-BCOR complexes ($p=0.0026$, $p=0.0014$, and $p=0.019$ respectively, Mann-Whitney) (Figure S1T). Similar effects were observed at both 24 and 48 h (Figure S1U–V). These results were confirmed in three additional independent mRNA-seq experiments in DLBCL cells after BCL6 vs. control siRNA knockdowns (Figure S1W–Z). Derepressed genes with BCL6 ternary complexes were also most significantly enriched in gene categories linked with the canonical and biologically validated BCL6 functions (Basso et al., 2004; Ci et al., 2009) including B-cell differentiation, B-cell activation, DNA replication, genes induced by STAT3 (Lam et al., 2008) and genes repressed by BCL6 in independent datasets(Shaffer et al., 2000) (Figure 1E). Hence ternary promoter complexes are most strongly linked to active repression by BCL6 and to canonical BCL6 biological functions.

BCL6 forms an obligate homodimer with two symmetric lateral grooves and so could theoretically bind to BCOR and SMRT simultaneously. To determine if BCL6 forms a true ternary complex we performed sequential ChIP (ChIP-re-ChIP) using BCOR or SMRT antibody followed by a second immunoprecipitation switching the two antibodies or using IgG control. We then performed QPCR to enrich promoter binding sites with overlapping BCL6/BCOR/SMRT peaks (*CD69*, *BANK1*, *PRDM1*, *TLR4*, and *CCR6* shown in Figure 2A–B and Figure S2A). In each case, sequential immunoprecipitation enriched these loci consistent with formation of ternary BCL6-SMRT-BCOR complexes (Figure 2C). As a positive control we performed ChIP-re-ChIP with BCL6 antibody followed by BCOR or SMRT ChIP (Figure S2B). To further confirm ternary binding we performed FRET (Fluorescence Resonance Energy Transfer) assays in which the BCL6 BTB homo-dimer

(Stogios et al., 2005) and fluorescent BCOR and SMRT BCL6 binding polypeptides were placed together in solution (Ahmad et al., 2003; Ghetu et al., 2008). This experiment resulted in a FRET signal, indicating that BCOR and SMRT fragments bind simultaneously to the homodimer (Figure 2D), as illustrated in Figure 2E. At higher concentrations of BCL6 BTB dimer, the majority of the peptides exist as single corepressor peptide/BCL6 BTB complexes, which produce no FRET signal (Figure 2D). Hence the BCL6 BTB dimer is able to coordinate assembly of a multifunctional ternary corepressor complex at gene promoters including both the PRC1-like BCOR and the HDAC3 containing SMRT complex.

BCL6 repression is linked to specific chromatin states and RNA Pol II pausing

In order to understand the chromatin context within which BCL6 is functional as a repressor we performed ChIP-seq for the H3K4me3, H3K9ac, H3K79me2, H3K36me3 activation marks, the H3K27me3 repressive mark and ERRBS for cytosine methylation in DLBCL cells. We then used an unbiased analysis approach (multidimensional principal component analysis), to group gene promoters according to the naturally occurring binding patterns of BCL6, corepressors, histone modifications and cytosine methylation (Figure 3A). We found that genes linked to principal component 2 (PC2) featured significantly lower transcript levels in DLBCL cells ($p<1e-8$) and most importantly, significant derepression after BCL6 siRNA ($p<1e-8$, Figure 3B). PC2 promoters were significantly enriched for BCL6, SMRT and BCOR as well as repression marks H3K27me3 and cytosine methylation, but at the same time were markedly depleted of all four active histone marks. In contrast, PC1 captured active genes associated with binding but not repression by BCL6. Overall, the PCA analysis indicated that only promoters with ternary complexes plus a completely repressed chromatin configuration are actively repressed by BCL6. BCL6 does not appear to be functionally significant at promoters with activation marks or where BCL6 is not forming a ternary complex.

Analysis of promoter ChIP-seq profiles further indicated that BCL6 binding occurred within the nucleosome free region (NFR) located just upstream of the transcriptional start site (TSS) as revealed by the valley of low H3K4me3 abundance (Figure S3A). SMRT and BCOR were precisely overlapped with BCL6 except that BCOR extended further downstream of the TSS, where RNA Pol II is localized in DLBCL cells. Indeed, ChIP-seq for paused (phosphoSer5) and elongating (phosphoSer2) RNA Pol II in DLBCL cells revealed that BCL6 repressed genes had a significantly higher paused versus elongating Pol II ratio compared to non-repressed BCL6 targets ($p<1e-8$, Figure 3C and S3C). This was independently confirmed by analyzing the distribution of total RNA pol II by ChIP-seq in DLBCL cells ($p<1e-8$ Figure S3B). Altogether, potent BCL6 repression of promoters in B-cells is linked to ternary BCL6-SMRT-BCOR corepressor complex formation within a specific chromatin context featuring loss of activating and gain of repressive marks, and suppression of RNA-pol II elongation but not Pol II recruitment (Figure S3D).

BCL6-SMRT complexes inactivate B-cell enhancers to repress proximal gene expression

Most BCL6-SMRT binding (85%) occurred outside of promoters suggesting that BCL6 mechanism may differ at these sites, perhaps linked to enhancer regions (Figure 4A). Enhancers are characterized by the presence of H3K4me1 and absence of H3K4me3 (Heintzman et al., 2009; Heintzman et al., 2007). We therefore performed H3K4me1 ChIP-seq to map enhancer regions in DLBCL cells. The vast majority of BCL6-SMRT distal/intronic peaks were $H3K4me3^{NEG}/H3K4me1^{POS}$ (n=2162) suggesting that these complexes are within transcriptional enhancers (Figure 4A). We first focused on distal BCL6-SMRT enhancer binding sites (n=818, >5kb away from TSSs). BCL6 and SMRT peak summits were precisely colocalized at enhancers, and generally restricted to a narrow region of less than 400bp framed by two adjacent nucleosomes as indicated by H3K4me1 read density

(Figure 4B). These BCL6-SMRT enhancers were significantly conserved as compared to adjacent control regions, which is suggestive of their functional relevance (Figure S4A).

We next examined whether BCL6-SMRT binding to enhancers has a cis-regulatory function. Since most BCL6-SMRT enhancers were located within 80kb from the nearest transcript (Figure S4B), we identified the most proximal gene for every BCL6-SMRT distal enhancer (n=553). Using GSEA we found that the group of genes with BCL6-SMRT bound enhancers were significantly enriched in genes derepressed after BCL6 knockdown (FDR=0.005; 24 h and FDR=0.03 at 48 h, Figure 4C and S4C). In contrast genes associated with distal enhancers bound by BCL6 without SMRT (n=654) were not enriched among BCL6 siRNA derepressed genes (FDR=0.38; 24 h and FDR=0.68 at 48h, Figure 4C and S4C). Similarly, BCL6-SMRT enhancer linked genes (but not BCL6 only) were significantly upregulated after BCL6 knockdown (BCL6-SMRT: $p<0.0001$ at 24h and $p=0.032$ at 48h, BCL6 only: $p=0.07$ at 24 h and $p=0.49$ at 48 h, Mann-Whitney U) compared to control genes (Figure 4D and S4D).

To further investigate whether BCL6 can repress through enhancer binding we performed reporter assays using constructs containing a BCL6-SMRT enhancer identified by our ChIP-seq, located 13kb upstream of the *CDKN1A* promoter and containing a BCL6 consensus binding motif (Figure 4E and S4E). Addition of *CDKN1A* distal enhancer induced 3-fold repression of *CDKN1A* promoter when transfected in DLBCL cells, and this repressor activity was markedly attenuated by BCL6 knockdown ($p<0.0001$, Mann-Whitney U, Figure 4F). Enhancer with mutated BCL6 binding site was unable to repress luciferase activity and instead enhanced *CDKN1A* promoter activity (Figure 4F). BCL6 knockdown did not induce higher expression from the mutant reporter. In 293T cells the *CDKN1A* distal enhancer acted as an inducer of transcriptional activity (Figure S4F). However, transfection of BCL6 (but not control plasmid) suppressed this *CDKN1A* enhancer activity. Collectively these data support the notion that BCL6 can repress enhancer elements.

BCL6 recruitment of SMRT deacetylates H3K27 to repress enhancers

Active enhancers can be distinguished from inactive or “poised” enhancers based on the presence of H3K27 acetylation (Creyghton et al., 2010; Rada-Iglesias et al., 2011). We performed H3K27ac ChIP-seq in DLCBL cells and observed that also in these cells, enhancers with high levels of H3K27ac are associated with highly expressed genes whereas enhancers with low H3K27ac level are associated with lower gene expression ($p<0.0001$, Mann-Whitney U, Figure S5A). Given the role of H3K27ac in enhancer activation, we hypothesized that BCL6 mediated recruitment of SMRT complex (which contains HDAC3) might deacetylate H3K27 thus rendering these enhancers inactive. QChIP assays were performed to detect H3K27ac at BCL6-SMRT enhancers, BCL6-only enhancers, or control loci in DLBCL cells transfected with either BCL6 or control siRNA. BCL6 knockdown increased the relative abundance of H3K27ac at most BCL6-SMRT enhancers but not at BCL6-only enhancers or control loci (Figure 5A). Accompanying the increase in H3K27 acetylation, BCL6 siRNA resulted in reduction of SMRT recruitment to BCL6-SMRT enhancers (Figure S5B), which paralleled the reduction in BCL6 enrichment (Figure S5C).

Because SMRT complexes contain HDAC3, we hypothesized that this histone deacetylase mediates H3K27 deacetylation. We therefore performed an *in vitro* HDAC assay using immunoprecipitated SMRT and HDAC3 complexes from DLBCL protein extract incubated with bulk histones, followed by immunoblotting for H3K27ac. This procedure yielded a marked decrease in H3K27ac among histones incubated with SMRT or HDAC3 complexes but not in IgG control pulldowns (Figure 5B). H3K27 deacetylation was abrogated by addition of the HDAC inhibitor trichostatin A (Figure 5B). To further explore the impact of HDAC3 on H3K27 acetylation in B-cells, we isolated splenic B-cells from mice with

conditional B-lineage specific deletion of *Hdac3* vs. littermate controls. We confirmed reduction of Hdac3 in conditionally deleted B-cells by western blotting and observed a reciprocal global increase of the H3K27ac compared to B-cells from control mice (Figure 5C).

To test whether disruption of the BCL6-SMRT complex could toggle enhancers to an active state, we treated DLBCL cells with the BCL6 small molecule inhibitor 79-6[1085], which blocks recruitment of corepressors to the BTB domain (Cerchietti et al., 2010a). 79-6[1085] caused the induction of H3K27ac at BCL6-SMRT enhancers but not at enhancers bound by BCL6 alone (Figure 5D). These effects are not due to loss of BCOR since BCOR complex did not deacetylate H3K27 (Figure S5D) nor did BCOR siRNA knockdown induce H3K27 acetylation levels at BCL6 target enhancers Figure S5E–F). Collectively these data suggest that BCL6 recruitment of SMRT results in HDAC3 dependent H3K27 deacetylation of enhancers and gene silencing. By disrupting BCL6 corepressor complexes BCL6 inhibitors can reactivate the BCL6 repressed enhancer network.

SMRT corepressor complexes antagonize p300 enhancer acetylation and activation

The p300 histone acetyltransferase (HAT) mediates H3K27 acetylation and enhancer activation(Jin et al., 2011; Visel et al., 2009). We hypothesized that BCL6-SMRT complexes would antagonize enhancer activation by p300. We performed p300 ChIP-seq in DLBCL cells and identified a total of 988 p300-bound enhancers. 87% (856/988) of these enhancers were H3K27ac^{HIGH}. We identified 369 enhancers with BCL6-SMRT only, 449 with BCL6-SMRT and p300, and 250 with BCL6-p300, raising the possibility that p300 and SMRT might compete for control of certain BCL6 target enhancers. Indeed we observed significantly lower levels of H3K27ac in BCL6-SMRT enhancers without p300 ($p<0.0001$, Mann-Whitney U) and significantly higher levels of H3K27ac in enhancers with BCL6 and p300 but without SMRT ($p<0.0001$) compared to enhancers that were occupied by BCL6 with both SMRT and p300 (Figure 6A).

In order to more globally evaluate the equilibrium between BCL6-SMRT complex and p300 on H3K27ac levels we performed H3K27ac ChIP-seq in cells treated with BCL6 or control siRNA (Figure S6A). Consistent with a role for SMRT in antagonizing p300 mediated H3K27ac, BCL6-SMRT enhancers without p300 displayed a greater increase in H3K27ac ($p<0.0001$, Mann Whitney U) compared to BCL6-SMRT enhancers that also contained p300 (Figure 6B). Moreover, BCL6-SMRT-p300 enhancers in turn featured greater induction of H3K27ac than BCL6 enhancers with p300 but without SMRT ($p<0.0001$). The greater increase of H3K27ac levels especially in BCL6-SMRT enhancers suggests that upon loss of BCL6-SMRT binding, p300 complexes can more efficiently acetylate H3K27.

As a complementary and unbiased approach to determine the link between gene expression and enhancer BCL6 complexes we performed a multidimensional PCA of distal enhancer BCL6 peaks. Genes associated with one principal component (PC3 n=715 genes) were notably derepressed upon BCL6 siRNA ($p<1e-8$, t-test). Consistent with the above data PC3 featured strong enrichment of BCL6, SMRT and H3K4me1, but no enrichment for H3K27ac or p300 (Figure 6C). In contrast PC1 and PC2 genes contained enhancer BCL6 complexes plus p300 with or without enhancer marks respectively, and were not strongly associated with genes repressed by BCL6. We repeated these analyses on the intronic BCL6-SMRT enhancers (n=1344) and observed a comparable association of BCL6-SMRT intronic enhancers with gene derepression, p300 binding and H3K27ac levels (Figure S6B–E). These data were validated using independent BCL6 siRNA knockdown RNA-seq replicates as well as additional enhancer histone mark ChIP-seq datasets including H3K4me2 which also marks enhancer regions (Ernst et al., 2011) (Figure S6F–L). These results suggest that BCL6 recruitment of SMRT/HDAC3 complexes to distal and intronic enhancer regions represses

gene expression by deacetylating H3K27ac and opposing the actions of p300 HAT complexes.

Altogether, the data suggest that BCL6 mediates its key biological effects in B-cells through at least two biochemically distinct BTB domain-dependent transcriptional repression mechanisms, repressing promoters most potently through multifunctional ternary complexes containing BCOR and SMRT, and repressing enhancers through SMRT-HDAC3 actions on H3K27ac (Figure 7). Both of these functions can be therapeutically targeted by BCL6 BTB domain peptide and small molecule inhibitors to kill DLBCL cells or suppress GC formation. Indeed exposure of DLBCL cells to RI-BPI resulted in the same preferential derepression of BCL6 ternary complex promoters and BCL6-SMRT enhancer associated genes as observed with BCL6 siRNA (Figure S6M–N).

DISCUSSION

Herein we report a unique mechanism through which a single transcription factor can serve as scaffold for recruiting structurally and functionally distinct chromatin modifying complexes through binding to identical surface motifs. We show that BCL6 simultaneously recruits both BCOR and SMRT/NCOR corepressors to symmetrical lateral grooves to form a ternary core repressor complex with BCL6 BTB domain homodimers. Yet SMRT and BCOR differ in their disposition around BCL6 regulated promoters. SMRT localizes focally with BCL6 at nucleosome free regions, whereas BCOR tends to spread downstream of the transcription start site. BCOR downstream spreading may be linked to our observation that BCL6 suppresses RNA Pol II elongation more than preventing loading of Pol II complexes. Repression through promoter ternary complexes is functionally linked to specific epigenetic chromatin marks associated with corepressor enzymatic activities (Gearhart et al., 2006; You et al., 2013).

At enhancers BCL6-SMRT complexes mediate silencing through a new mechanism involving HDAC3 deacetylation of H3K27. SMRT recruitment appears to compete with enhancer activation mediated by p300 through H3K27 acetylation, thus providing a basis for dynamic and reversible “toggling” of enhancers. This would be different from the effect of the histone demethylase LSD1, which permanently erases enhancers through H3K4 demethylation (Whyte et al., 2012). Nonetheless, it remains to be investigated how H3K27 acetylation is linked to enhancer activity. Enhancer toggling may play a physiological role in enabling recycling of B-cells between the dark zone and light zone of GCs. Transient interactions with T-cells in the light zone triggers CD40 and MAPK signaling in B-cells, which phosphorylates and delocalizes SMRT and NCOR to the cytoplasm, leading to reversible derepression of BCL6 targets (Polo et al., 2008; Ranuncolo et al., 2007). Presumably CD40 toggling of BCL6 enhancers enables B-cells to become competent for terminal differentiation if they have generated a high affinity immunoglobulin, or to undergo apoptosis if they are damaged or unable to form high affinity antibody. Toggling back to the repressed state permits recycling of B-cells to the dark zone for additional rounds of affinity maturation. Along these lines it was shown that once CD40 signaling is disengaged, SMRT returns to BCL6 and BCL6 target gene repression is restored (Polo et al., 2008). In support of this notion, analysis of genes that are upregulated in GC light zone B-cells (centrocytes) as compared to dark zone cells (centroblasts)(Caron et al., 2009) show significant upregulation of GC B-cell BCL6-SMRT enhancer related target genes but not BCL6-only enhancers genes ($p<0.0001$, Mann Whitney U, Figure S6O–P). BCL6-SMRT enhancer targets were also significantly enriched among centrocyte-upregulated genes (FDR=0.006, GSEA). Moreover, CD40 signaling and MAP kinase pathways are strongly enriched among genes regulated by BCL6-SMRT enhancer complexes (Figure S6Q).

Enhancer toggling may be pathologically suppressed in certain DLBCLs containing *EP300* inactivating mutations (Cerchietti et al., 2010b; Pasqualucci et al., 2011). Reduction in EP300 function could tip the balance of transcriptional repression in favor of BCL6-SMRT complexes and thus favor the oncogenic effects of BCL6. BCL6 BTB blockade was sufficient to induce H3K27ac levels at BCL6-SMRT target enhancers. Hence enhancer toggling by BCL6 inhibitors may contribute to their anti-lymphoma effects (Figure 7).

BCL6 ternary complex and BCL6 enhancer complexes seem to be independent of each other, since there was no trend towards overlap at the same genes ($p=0.957$) and no tendency for the small set of overlapping promoter-enhancer complex containing genes to be more derepressed after BCL6 siRNA ($p=0.44$, Mann Whitney test, data not shown). Specific BCL6 target gene sets may thus be independently controlled through its two different BTB domain dependent repression mechanisms. Collectively the BTB-dependent mechanisms we identified are essential for DLBCLs and the normal GC B-cells from which they are derived (e.g. as in Figure 1A and S1N). However our data do not rule out that other BCL6 repression mechanisms may exist and contribute in some way to its actions in B-cells or other cell types (Mendez et al., 2008; Parekh et al., 2007). Further research into the biochemistry of BCL6 in B-cells and other cell types is warranted to explore this question. It is notable that BCL6 was also shown to be localized at enhancers in macrophages (Barish et al., 2012). However BCL6 functions at macrophage enhancers actions are likely mechanistically different than B-cells since BTB domain dependent corepressor recruitment is dispensable for the actions of BCL6 in this cell type (Huang et al., 2013).

In summary, our data highlight the flexibility of BCL6 to simultaneously regulate gene expression through different mechanisms on different gene sets within the same cells, through the same protein interface. From the immunology perspective it is notable that these mechanisms are specifically significant to B-cells but do not play a major role in the actions of BCL6 in T-cells or macrophages. Hence BCL6 displays a tremendous degree of flexibility and complexity in the immune system. Importantly therapeutic targeting of BCL6 with inhibitors that block the BTB lateral groove results in simultaneous blockade of both BTB dependent mechanisms, but has no effect on other compartments of the immune system. This enables cell type specific inhibition of BCL6 in lymphomas and B-cells without needing to resort to complicated tissue-specific delivery systems. Finally, although our current studies have focused on BCL6, it is likely that enhancer toggling and biochemical functional diversity are more general mechanisms relevant to other enhancer transcription factors.

EXPERIMENTAL PROCEDURES

Chromatin Immunoprecipitation

OCI-Ly1 or purified GC B-cells were fixed, lysed and sonicated to generate fragments less than 400bp. Sonicated lysates were incubated with antibodies overnight (Supplemental Information) and after increasing stringency washes immunocomplexes were recovered and DNA was isolated. ChIP and input DNA was used in Q-PCR reactions to estimate relative enrichment. In experiments using drug treatments (Figure 5D) cells were treated with compounds (50uM) for 30min and after completion of the assay ChIP and input DNA were quantified using Qubit 2.0 fluorometer (Invitrogen) so that an equal amount of DNA was added to each PCR reaction.

ChIP-re-ChIP

Experiments were performed as above. After the first round of ChIP, immunocomplexes were eluted by incubating the beads in 50ul TE buffer supplemented with 10mM DTT and

protease inhibitors for 30min at 37oC rocking. The eluted immunocomplexes were diluted up to 1mL with dilution buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl, protease inhibitors) and antibodies were added for a second round of ChIP. Finally the bound DNA was eluted and enrichment was quantified by Q-PCR and gel electrophoresis of PCR products.

ChIP-seq

ChIP-seq libraries were prepared using the Illumina ChIP-seq Library preparation Kit following the manufacture's instructions with minor modifications starting with 10ng of purified ChIP DNA (See Supplemental information). An input chromatin control library was generated for each ChIP-seq experiment starting from the same amount of material and was used as a negative control for peak calling and downstream analyses using the ChIPseeqer package (Giannopoulou and Elemento, 2011). Details on Illumina data analysis and number of detected peaks can be found in the Supplemental information.

Gene expression analysis by mRNA-seq

Three ug of total RNA was isolated from at 24 h and 48 h after siRNA nucleofection. RNAeasy Plus Kit (Qiagen) that included a gDNA elimination step was used for RNA isolation. RNA concentration and purity were determined using Nanodrop (Thermo Scientific) and integrity was verified using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Libraries were generated using mRNA-seq sample prep kit (Illumina). Briefly, mRNA was selected by two rounds of purification using magnetic polydT beads and then fragmented. First strand synthesis was performed using random oligos and SuperscriptIII (Invitrogen). After second strand synthesis a 200bp paired-end library was prepared following the Illumina paired-end library preparation protocol.

Statistical analysis

Two-tailed Mann-Whitney U test was used unless otherwise stated. For details on PCA analysis see Supplemental Methods. All statistical analyses were carried out using Prism software (Graphpad) and R statistical package.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- BCL6 most potently represses promoters through a ternary complex with BCOR and SMRT.
- BCL6 represses enhancers by recruiting SMRT-HDAC3 complexes to deacetylate H3K27.
- BCL6 can “toggle” enhancers to a poised state, which can be reversed by p300.

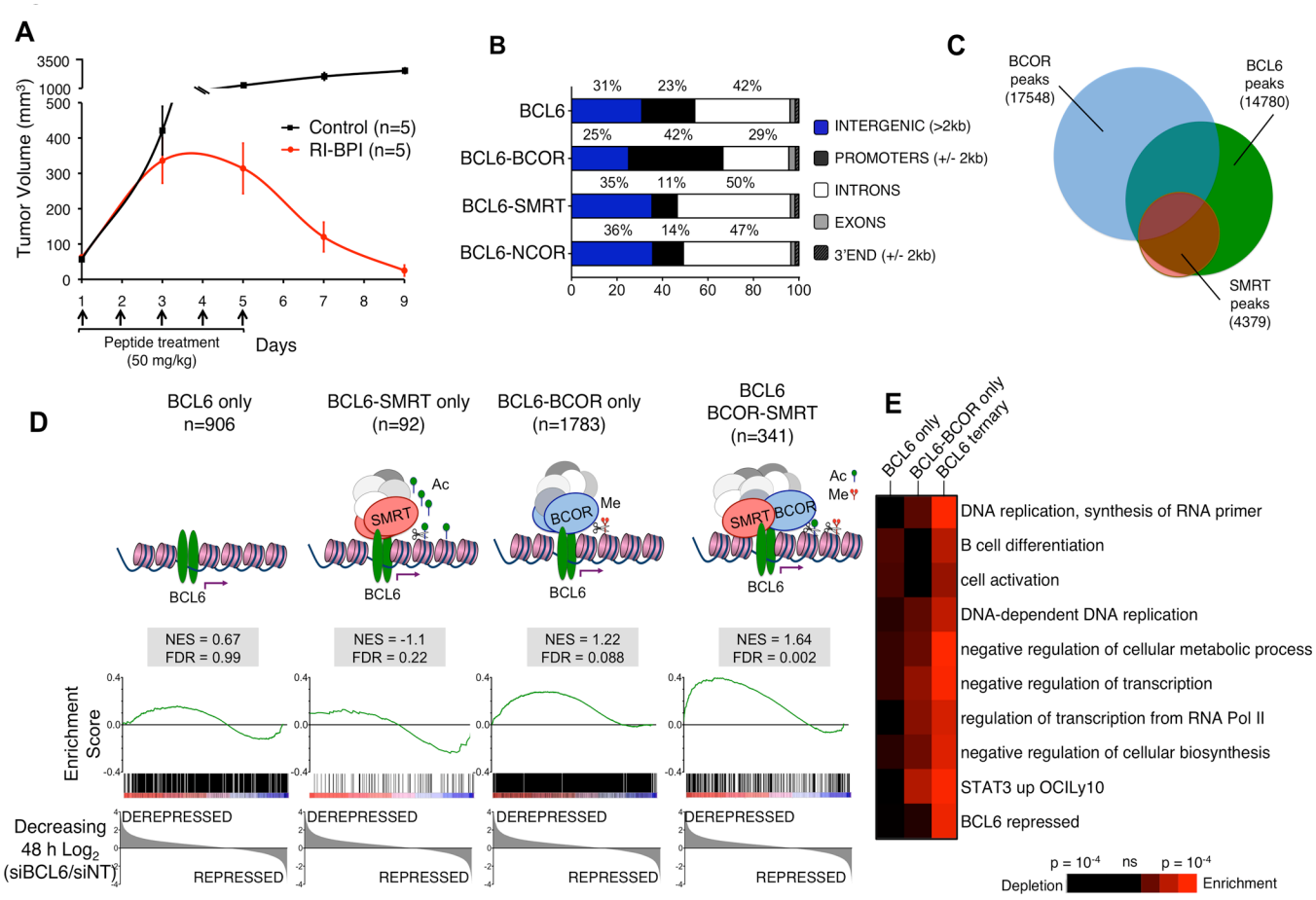

Figure 1.

Genome wide distribution of BCL6 and corepressors BCOR, SMRT and NCOR in DLBCL cells. (**A**) Tumor growth plot in DLBCL xenografted mice (Farage cell line) after treatment with RI-BPI vs control peptide (50 mg/kg/day for 5 consecutive days) (**B**) Genomic distribution of BCL6 peaks and BCL6 peaks coinciding with BCOR (BCL6-BCOR), SMRT (BCL6-SMRT) and NCOR (BCL6-NCOR) peaks based on their location relative to RefSeq transcripts (hg18) in OCI-Ly1 cells. (**C**) Venn diagrams representing the overlap of BCL6, BCOR and SMRT ChIP-seq peaks in DLBCL cells. (**D**) GSEA analysis integrating ChIP-seq and mRNA-seq results after BCL6 knockdown. The enrichment of promoter target genes bound by BCL6 ternary complexes (BCL6-BCOR-SMRT), BCL6-BCOR only, BCL6-SMRT only, or BCL6 only was tested based on decreasing gene all expression log ratios (48 h; siBCL6/siNT). Weighed statistic and 5000 sample permutations were used. NES: Normalized enrichment score, FDR: False Discovery Rate. (**E**) Pathway analysis using BCL6-ternary target genes (n=126) upregulated more than 1.5 fold after BCL6 knockdown. See also Figure S1 and Table S1.

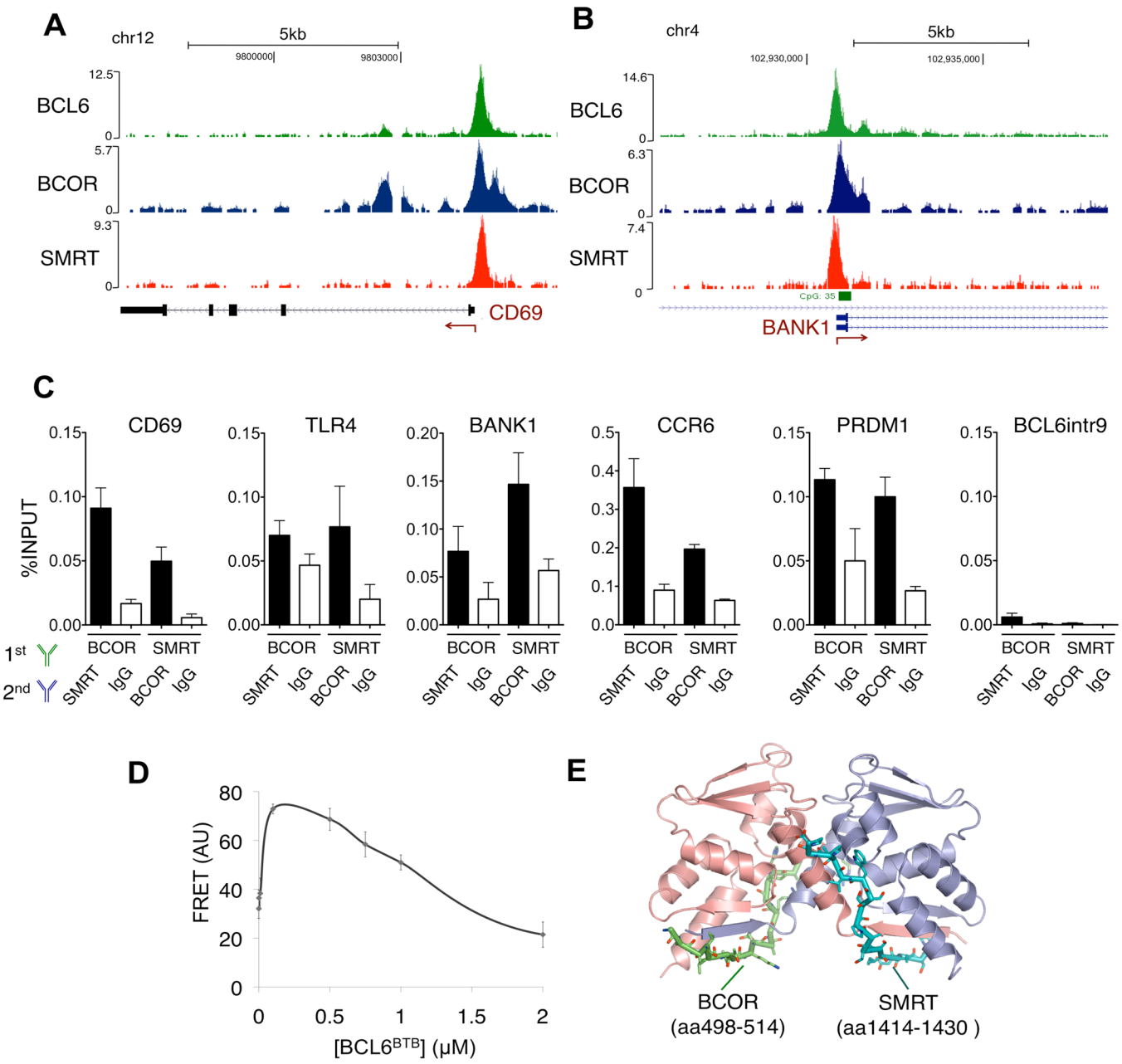

Figure 2.

BCL6 can recruit both BCOR and SMRT corepressor complexes simultaneously through homodimerization of its BTB domain in promoters. (**A–B**) BCL6 binding (green) at the promoters of *CD69* and *BANK1* coincides with binding of corepressors BCOR (blue) and SMRT (red) respectively. y-axis values represent read densities normalized to total number of reads. (**C**) ChIP-re-ChIP assay in BCL6-BCOR-SMRT promoters using BCOR and SMRT antibodies. IgG was used as a negative control. A *BCL6* intron 9 locus is shown as a negative control. Experiment was performed in duplicate using triplicate wells. The y-axis depicts enrichment as % input +/−SEM. (**D**) FRET assay for A488-BCOR and BODIPY-SMRT peptides in solution with BCL6 BTB. Fluorescence (AU) is shown as a function of increasing BCL6 BTB concentration. FRET emission is generated when both peptides bind to the BCL6 BTB dimer. Higher concentrations of BCL6 BTB dimers increase single

peptide binding evens decreasing FRET emission. (**E**) Hybrid model of the BCL6 BTB dimer (each monomer in violet and pink) simulated in complex with two peptides corresponding to BCOR 498–514 (green) and SMRT 1414–1430 (cyan) used in FRET assay. See also Figure S2.

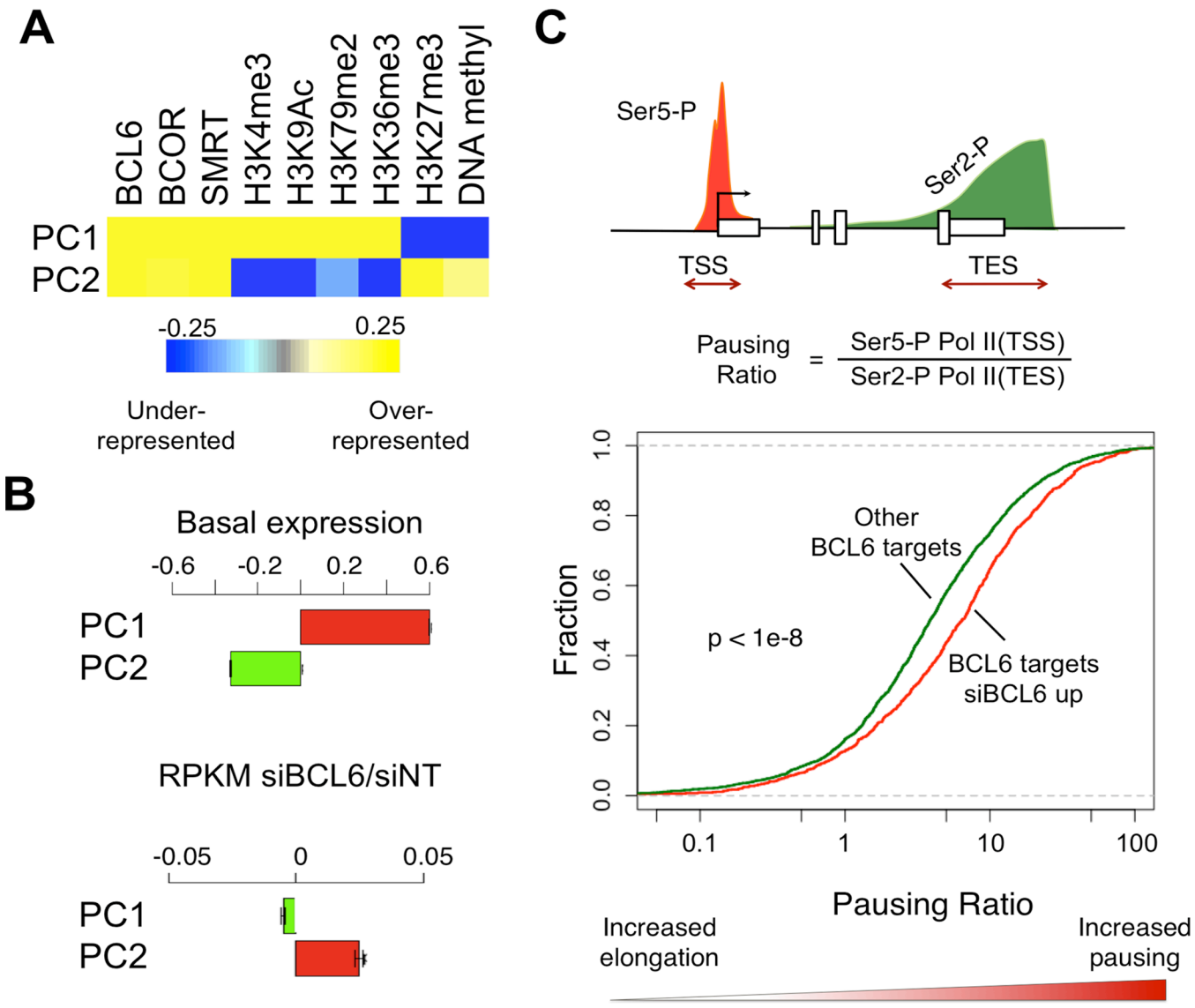

Figure 3.

Potent BCL6 repression occurs within repressed chromatin states and is linked to RNA Pol II pausing.

(**A**) Graphical representation of weighted PCA analysis integrating histone mark and DNA methylation enrichment levels surrounding all TSS. The top two PCs are shown. Color key indicates the weights of the original variables assayed by ChIP-seq and ERRBS. (**B**) Correlation of genes corresponding in each PC with basal levels of gene expression and gene expression changes after BCL6 knockdown (48h) is indicated. (**C**) Cumulative distribution of RNA pol II Pausing Ratios (calculated as the fraction of normalized read density ratio of Ser5-P Pol II (paused) around the TSS (−100 to +200bp) to the Ser2-P Pol II (elongating) density at the TES (TES+2kb)) comparing BCL6 target genes upregulated after BCL6 siRNA versus the rest of BCL6 target genes. p-value is indicated. See also Figure S3.

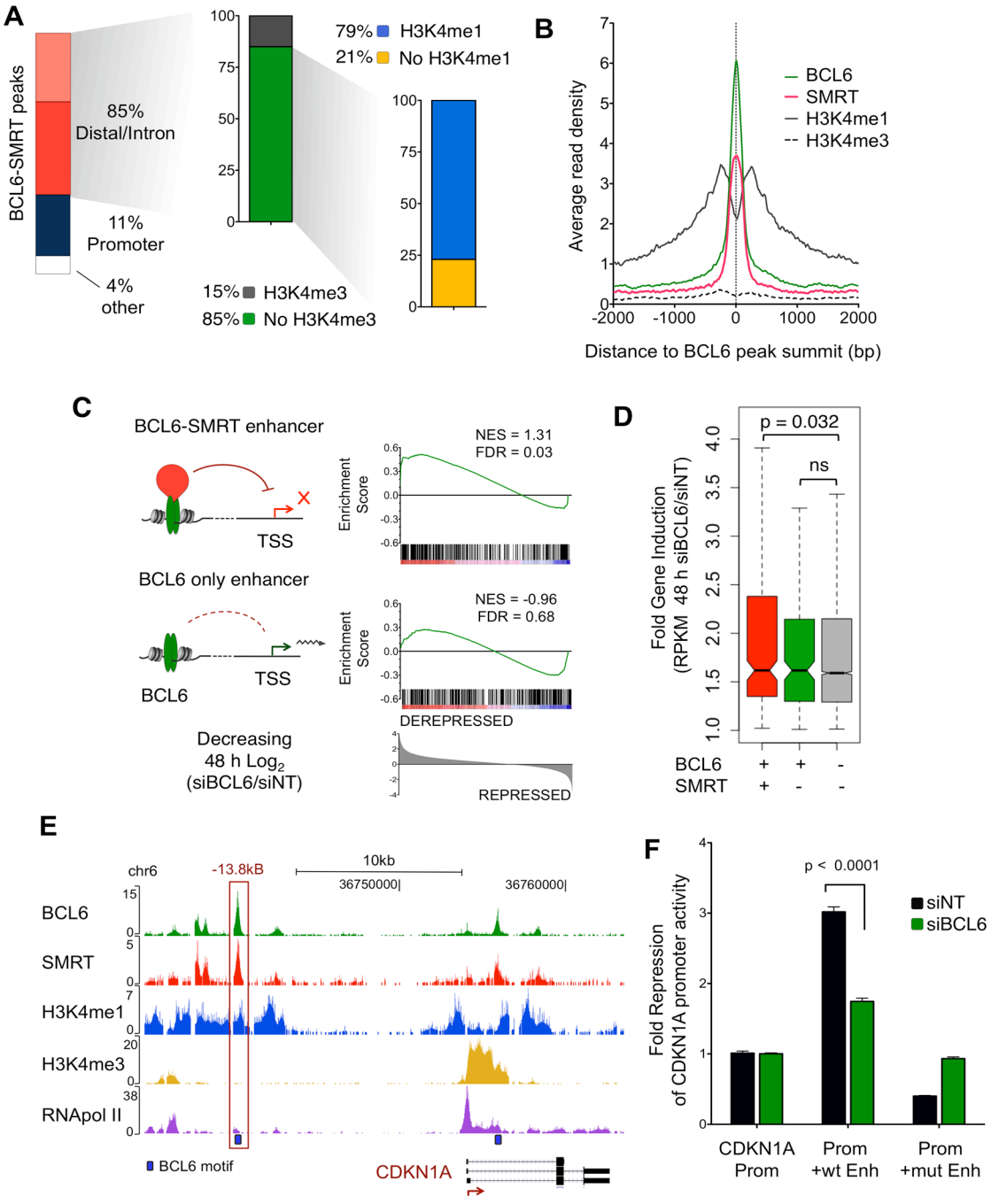

Figure 4.

BCL6-SMRT complexes mediate enhancer silencing (**A**) Overlap of distal/intronic BCL6-SMRT peaks with H3K4me3 and H3K4me1 peaks in DLBCL cells. (**B**) BCL6, SMRT and H3K4me1,3 ChIP-seq read density profiles in BCL6-SMRT enhancers centered at the BCL6 peak summit. y-axis represents average read densities normalized to total number of reads. (**C**) GSEA analysis of genes proximal to BCL6-SMRT bound enhancers or BCL6 non-SMRT enhancers. Genes were ranked based on decreasing $\log_2$ RPKM (siBCL6/siNT) at 48 h BCL6 knockdown (weighted p2 statistic, 5000 permutations). (**D**) Comparison of fold expression induction (siBCL6/siNT RPKM, 48 h) of genes proximal to BCL6-SMRT

enhancers or BCL6 non-SMRT enhancers versus other genes. (**E**) A BCL6-SMRT enhancer located −13kb upstream of *CDKN1A* is illustrated. UCSC tracks of BCL6, SMRT, H3K4me1,3 and total RNA polymerase II density normalized to the total number of reads are represented. Location of BCL6 sequence motifs is indicated (**F**) Reporter assays performed in DLBCL cells with constructs containing *CDKN1A* promoter alone (Prom), promoter and wild type −13kb enhancer (Prom + wt Enh) or promoter and mutant enhancer (Prom+ mut Enh). Cells were treated with siBCL6 and siNT as indicated in quadruplicates. Y-axis represents fold repression of reporter based on relative luciferase (vs. TK-Renlla) compared to the basal activity of the promoter construct. Data are represented as mean +/− SEM. See also Figure S4.

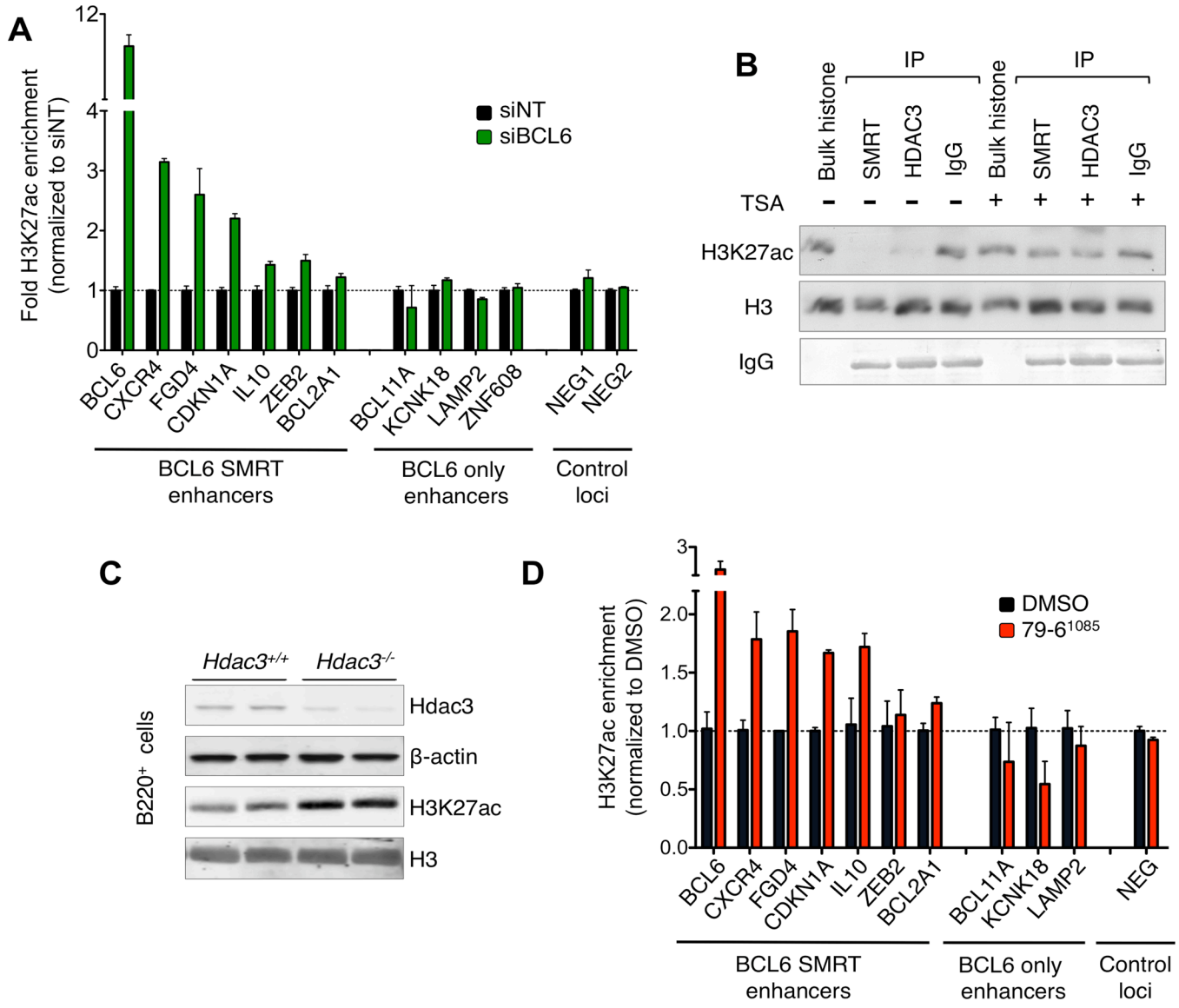

Figure 5.

BCL6 recruits SMRT to deacetylate H3K27 leading to enhancer inactivation. (**A**) Selected BCL6-SMRT bound enhancers or BCL6 only enhancers were tested for enrichment of H3K27ac by Q-ChIP in OCI-Ly1 cells nucleofected with siBCL6 or siNT. Relative enrichment is normalized to siNT and shown as mean +/− SEM (**B**) H3K27ac immunoblot of *in vitro* histone deacetylation reactions using immunoprecipitated SMRT and HDAC3 in the presence or absence of TSA (Trichostatin A). IgG was used as a negative IP control and H3 as a loading control. Coomassie stain indicates that equal amount of antibody was added in each IP. (**C**) H3K27ac immunoblot using whole cell extracts of B220+ cells isolated from two $Hdac3^{+/+}$/ROSA-GFP/CD19-Cre and two $Hdac3^{FL/-}$/ROSA-GFP/CD19-Cre mice. Hdac3 depletion in null cells was confirmed. H3 and β-globin were used as loading controls. (**D**) Biological replicates of H3K27ac QChIP performed in triplicates in OCI-Ly1 cells exposed to 50 μM of $79\text{-}6^{1085}$ or vehicle (DMSO) for 30 min. Fold H3K27ac enrichment vs. vehicle is shown (y-axis). Error bars represent the mean +/− SEM. See also Figure S5.

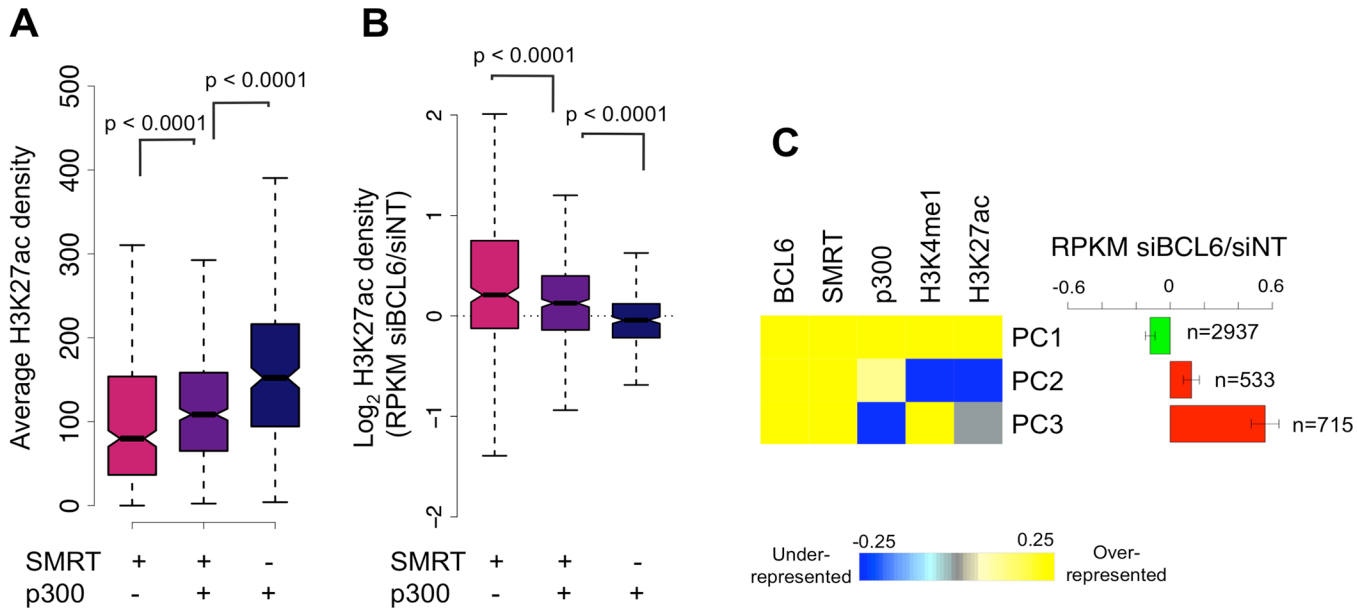

Figure 6.

BCL6-SMRT complexes antagonize p300 acetyltransferase activity to mediate enhancer toggling. (**A**) Comparison of the average normalized H3K27ac read density levels in BCL6 enhancers bound by SMRT but not p300, bound by both SMRT and p300 or bound by p300 but not SMRT (**B**) Log_2 change of H3K27ac levels upon BCL6 knockdown in BCL6 enhancers bound by SMRT but not p300, bound by both SMRT and p300 or bound by p300 but not SMRT. p-values are indicated (**C**) Graphical representation of weighted PCA analysis using distal BCL6 binding sites. Correlation with gene expression changes after BCL6 depletion (24 h) links PC3 (715 distal enhancers, $p < 1e\text{-}8$) to gene derepression. Color key indicates the weights of the original variables assayed by ChIP-seq. See also Figure S6.

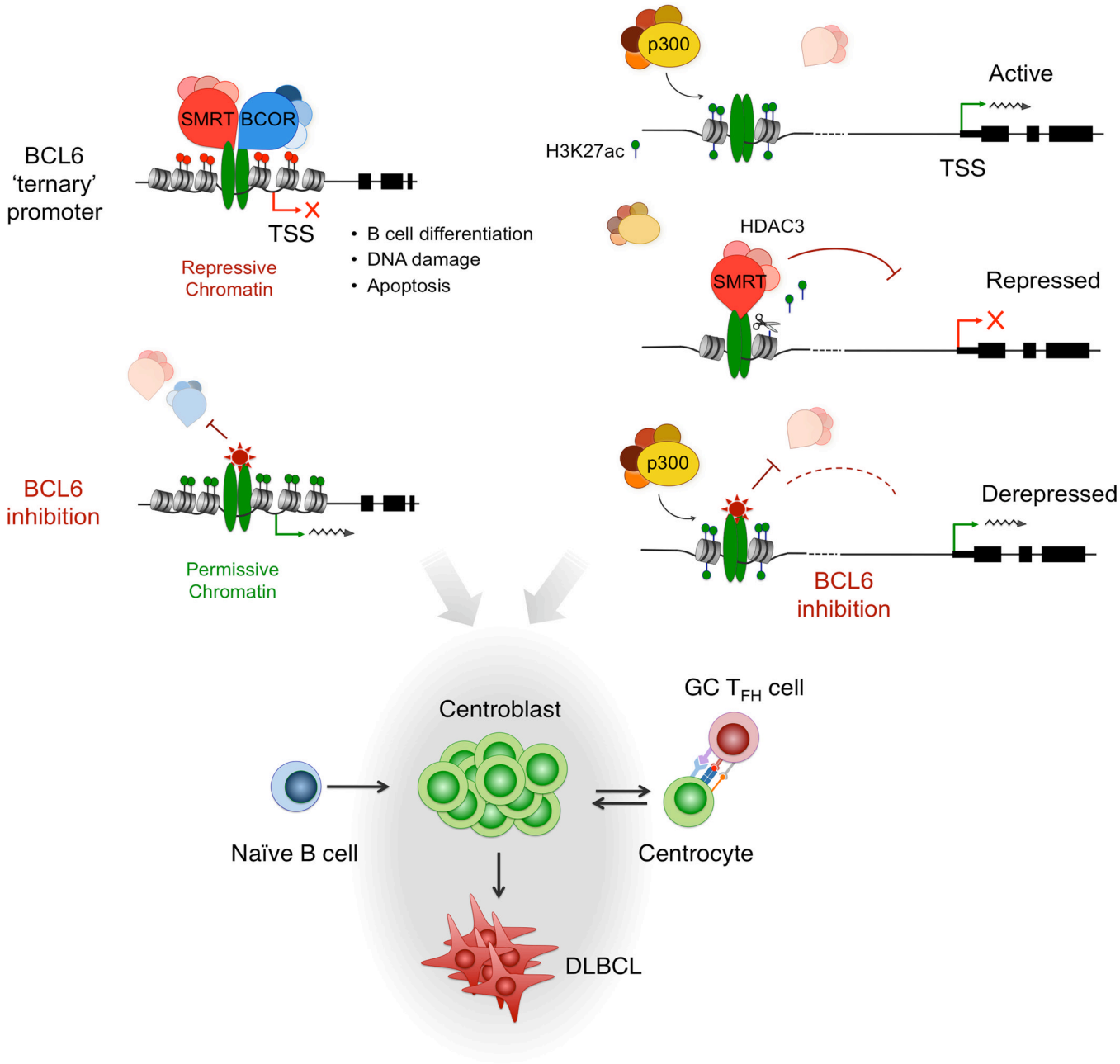

Figure 7.

Model of BCL6 repression mechanism. BCL6 dimers can simultaneously recruit PRC1-like BCOR complexes and HDAC3-containing SMRT complexes in B cell promoters to most effectively repress transcription in a repressed chromatin environment. Alternatively BCL6 selectively recruits SMRT to functionally inactivate a network of B cell enhancers through H3K27 deacetylation opposing the effect of p300 complexes that mediate H3K27 acetylation. BCL6 BTB inhibitors dismiss BCOR and SMRT complexes and reactivate the BCL6 targeted gene programs thereby killing lymphoma cells while BCL6-BTB point mutations abrogate GC formation.