Title:
Detection of Enhancer-Associated Rearrangements Reveals Mechanisms of Oncogene Dysregulation in B-cell Lymphoma

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Running Title:
Enhancer-Associated Rearrangements in Lymphoma

Keywords:
Lymphoma
Rearrangement
Enhancer
BCL6
MYC

Notes:
Russell Ryan received funding from NIH training grants T32GM007748 and T32CA009216.
Bradley Bernstein received funding from ENCODE, the Leukemia and Lymphoma Society, and the Howard Hughes Medical Institute.

The authors disclose no potential conflicts of interest.

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Abstract

B-cell lymphomas frequently contain genomic rearrangements that lead to oncogene activation by heterologous distal regulatory elements. We utilized a novel approach, termed ‘Pinpointing Enhancer-Associated Rearrangements by Chromatin Immunoprecipitation’ or PEAR-ChIP, to simultaneously map enhancer activity and proximal rearrangements in lymphoma cell lines and patient biopsies. This method detects rearrangements involving known cancer genes, including $CCND1$, $BCL2$, $MYC$, $PDCD1LG2$, $NOTCH1$, $CIITA$, and $SGK1$, as well as novel enhancer duplication events of likely oncogenic significance. We identify lymphoma subtype-specific enhancers in the $MYC$ locus that are silenced in lymphomas with $MYC$-activating rearrangements and are associated with germline polymorphisms that alter lymphoma risk. We show that $BCL6$-locus enhancers are acetylated by the $BCL6$-activating transcription factor MEF2B, and can undergo genomic duplication, or target the $MYC$ promoter for activation in the context of a “pseudo-double-hit” t(3;8)(q27;q24) rearrangement linking the $BCL6$ and $MYC$ loci. Our work provides novel insights regarding enhancer-driven oncogene activation in lymphoma.

Significance

We demonstrate a novel approach for simultaneous detection of genomic rearrangements and enhancer activity in tumor biopsies. We identify novel mechanisms of enhancer-driven regulation of the oncogenes $MYC$ and $BCL6$, and show that the $BCL6$ locus can serve as an enhancer donor in an “enhancer hijacking” translocation.

Introduction
Genomic rearrangements represent an important oncogenic mechanism in human cancers. Rearrangements occurring within genes may produce fusion transcripts encoding chimeric proteins with novel functions. In contrast, many recurrent translocations in B-cell lymphomas, such as those involving BCL2, CCND1, and frequently MYC, result in high-level expression of intact oncogene coding transcripts from their native promoters. Aberrant oncogene expression in these events is thought to be dependent on looping interactions between the oncogene promoter and cis-regulatory elements (enhancers) from the translocation partner locus, often IGH (1). The identification of recurrent “enhancer hijacking” translocations (2) and enhancer amplification events (3) in non-lymphoid cancers suggests that this may be a common oncogenic mechanism, and raises the need for improved methods for genome-wide detection and functional characterization of such events.

Enhancers are non-coding regulatory elements that stimulate transcription through looping-mediated interactions with promoters, and are activated in specific cellular contexts by different combinations of sequence-specific transcription factors (TFs). Active enhancers adopt a signature chromatin structure, and can be identified by mapping histone H3 Lys27 acetylation (H3K27ac) via chromatin immunoprecipitation and high-throughput sequencing (ChIP-Seq) (4,5). Strong histone acetylation is a common feature of genomic loci that undergo recurrent physiologic or oncogenic immunoglobulin gene rearrangements in B-cell lymphoma (6,7).

Here we describe PEAR-ChIP (Pinpointing Enhancer-Associated Rearrangements by Chromatin Immunoprecipitation and Paired-end sequencing), a novel approach that combines H3K27ac ChIP-Seq with paired-end sequencing analysis to map genomic rearrangements involving acetylated regulatory elements. Investigating a panel of 14 primary patient biopsies and 8 cell line models representing multiple classes of B-cell lymphoma, we identify known and novel rearrangements, and gain insight into
the mechanisms by which these translocations exploit native regulatory circuits to drive activation of
MYC, BCL6 and other oncogenes.

Results

Identification of oncogenic rearrangements in mantle cell lymphoma by PEAR-ChIP

Histone H3K27ac ChIP-Seq is a powerful tool for genome-wide identification of active enhancers, but
identifying relationships between enhancers and genomic rearrangements has required addition of a
second sequencing technology, such as whole-genome sequencing (WGS) (2). However, we reasoned that
analysis of paired-end sequencing data from H3K27ac ChIP-Seq libraries could efficiently detect
rearrangements involving enhancers, as long as the breakpoints occurred within acetylated elements (Fig.
1A).

We first tested this approach in mantle cell lymphoma (MCL), a poor-prognosis lymphoma characterized
by reciprocal translocations between the IGH J recombination region on chromosome 14 and a >300 kb
gene-free region upstream of the CCND1 gene on chromosome 11, with half of cases showing breakpoint
within a major translocation cluster (MTC) (8).

We performed H3K27ac ChIP-seq with paired-end sequencing on frozen tissue from four primary MCL
tumor biopsies (see Supplementary Table S1 for clinical and diagnostic details about all samples) and four
MCL cell lines. All cases showed strong H3K27ac signal extending from the IGH μ intronic enhancer and
covering the J recombination region. In each case, we identified sequencing read pairs that spanned the
t(11;14) rearrangement breakpoint, allowing for precise breakpoint identification (Fig. 1A, 1B).
Chromosome 11 breakpoints were visible in H3K27ac ChIP-Seq tracks as ‘spikes’ of acetylation signal in
the gene desert upstream of CCND1. In four MCL cases, atypical read pairing also identified a focal deletion or inter-chromosomal rearrangement resulting in partial deletion of the CCND1 3’ UTR, a recurrent event in MCL that increases stability of the CCND1 transcript by eliminating a microRNA binding site, and is associated with a more aggressive disease course (9). In all eight cases, we also detected productive VDJ recombination of the alternate IGH allele not affected by the t(11;14) (Fig. 1A, Supplementary Table S2).

To establish the genome-wide PEAR-ChIP method, we adapted dRanger and BreakPointer (10), originally developed for detecting rearrangements in WGS data, to scan paired-end H3K27ac ChIP-Seq data for genomic alterations. In the MCL line Rec-1, PEAR-ChIP detected a truncating deletion of NOTCH1, recently shown to be an activating oncogenic event (11). In the MCL line Jeko-1, we detected a rearrangement between an enhancer-rich region of chromosome 8p and the MYC locus at 8q24 (Fig. 1C). This event appears to have preceded the MYC locus amplification previously documented in this line (12), since high-level amplification at both sides of the breakpoint was evident in alignment data for a control sequencing library (data not shown). The ability of PEAR-ChIP to detect MYC rearrangements is of particular interest, given the association of these events with aggressive clinical behavior in multiple tumor types.

**PEAR-ChIP identifies known and novel oncogenic rearrangements in diverse B-cell lymphomas**

High-grade B-cell lymphomas (HGB), including diffuse large B-cell lymphoma (DLBCL) and related variants such as primary mediastinal large B cell lymphoma (PMBL), show remarkable genetic and epigenetic heterogeneity. We used PEAR-ChIP to profile four HGB cell lines and seven primary HGB biopsies, as well as three lymph node biopsies from patients with chronic lymphocytic leukemia / small lymphocytic lymphoma (CLL/SLL).
To evaluate the efficacy of PEAR-ChIP in detecting known oncogenic rearrangements, we compared the results from our four HGB cell lines to previously published whole genome sequencing data for those same lines (13). WGS detected a total of 6 inter-chromosomal rearrangements or large-scale inversions in these lines associated with a gene known to be recurrently targeted by structural rearrangements in DLBCL according to the Mitelman database. PEAR-ChIP detected all 6 events, including three involving BCL2, and three near MYC (Supplementary Table S3). Compared to WGS, PEAR-ChIP yielded 2-17 times more supporting reads for those rearrangements, despite a 10-31 fold lower total sequencing depth indicating that PEAR-ChIP can capture such rearrangements with one to two orders of magnitude less sequencing depth. H3K27ac PEAR-ChIP also detected breakpoints at the edges of many amplifications, deletions, and large-scale rearrangements at non-recurrent loci, although these represented only a fraction of the events detected by WGS. Inter-chromosomal rearrangements involving the IGH recombination region showed particularly deep coverage, likely due to strong acetylation extending from the nearby IGH μ intronic enhancer, with an average of 34 fragments per 10 million mapped read pairs spanning t(11;14) IGH-CCND1 rearrangement junctions, and an average of 139 fragments per 10 million mapped read pairs spanning t(14;18) IGH-BCL2 junctions. Although BCL6 rearrangements are not represented in our HGB lines, sequencing coverage was high over known breakpoint cluster regions in the BCL6 locus, with PEAR-ChIP datasets showing an average coverage depth of 69 (standard deviation of 32) fragments per 10 million mapped read-pairs over the 5.3 kb major translocation cluster region in the BCL6 promoter (14), and an average depth of 130 (standard deviation of 26) fragments per 10 million mapped read-pairs over the 31.2 kb alternate breakpoint region (15), which lies within a highly acetylated “super-enhancer” region upstream of BCL6 (16,17).

We next focused on large-scale rearrangements involving the MYC locus, which were identified by PEAR-ChIP in 4 cell lines (3 HGB and 1 MCL) and one HGB biopsy (Fig. 1C). Notably, all partner loci in these rearrangements showed strong candidate enhancers adjacent to the breakpoint by H3K27ac ChIP-seq.
signal. Both \textit{IGH} rearrangement breakpoints fell on the 5’ side of the \textit{MYC} gene as expected (18), while all non-\textit{IGH} rearrangement breakpoints occurred on the 3’ side, with the breakpoint in HGB-05 occurring >400 kb downstream of the \textit{MYC} promoter. One additional \textit{MYC} event, a t(3;8) rearrangement involving the \textit{MYC} and \textit{BCL6} loci in HGB-07, was detected by conventional cytogenetics and FISH, but not by PEAR-ChIP. Deeper sequencing of the HGB-07 control library allowed for localization of rearrangement breakpoints in this case, which occurred in non-acetylated regions, but still linked \textit{MYC} to a strong enhancer as discussed below.

PEAR-ChIP analysis of HGB biopsies detected large-scale rearrangements involving several other known translocation targets, including \textit{BCL2} (n=2), \textit{CIITA} (19), and \textit{PDCD1LG2} (20) (Fig 2A, 2B). The \textit{CIITA-IL4R} and \textit{PDCD1LG2-NCOA3} events involve novel translocation partners, with the latter event consisting of an unusual cryptic insertion of the \textit{PDCD1LG2} gene from chromosome 9 into the \textit{NCOA3} gene on chromosome 20. We also detected a t(6;14)(q22;q32) translocation between the \textit{IGHG3} switch region and an intron of \textit{SGK1} (Fig. 2B), likely resulting in \textit{SGK1} inactivation. \textit{SGK1} has not been previously reported as a translocation target, but is located in a region of recurrent genomic deletion, and is a frequent target of inactivating mutations in DLBCL (13). Another novel translocation joined the \textit{IGHV} segment region to a breakpoint 300 kb downstream of the transcriptional regulator \textit{ID2} (Supplementary Fig. S1A-F).

To evaluate the functional significance of PEAR-ChIP-detected rearrangements, we quantified the expression of putative target genes by qRT-PCR across 17 HGB biopsies (Fig. 2C), selected to include at least five cases each of germinal center B-cell (GCB)-DLBCL, activated B-cell (ABC)-DLBCL, and PMBL by immunohistochemistry (IHC), signature gene expression, and clinical criteria (Supplementary Fig. S2A). The anti-apoptotic gene \textit{BCL2} is silenced as part of the normal germinal center gene expression program; as expected, \textit{BCL2} expression was high in the two GCB-DLBCL biopsies with PEAR-ChIP-detected \textit{IGH-BCL2} rearrangements, but low in the other three (Fig. 2C). Similarly, \textit{PDCD1LG2} expression was markedly
higher in HGB-01, an ABC-DLBCL that harbors an \textit{NCOA3-PDCD1LG2} rearrangement, than in other DLBCL cases. RT-PCR confirmed expression of an \textit{NCOA3-PDCD1LG2} fusion transcript, as predicted by PEAR-ChIP (Supplementary Fig. S2B). High expression of \textit{PDCD1LG2} is typical of PMBL (21), where it is activated by JAK-STAT signaling and frequent genomic abnormalities (20), but is unusual in ABC- and GCB-DLBCL. We confirmed low relative expression of \textit{SGK1} and \textit{CIITA} in tumors where PEAR-ChIP detected inactivating rearrangements.

In addition to known rearrangement targets, two other genes showed multiple independent PEAR-ChIP-detected events in our datasets (Supplementary Fig. S1B-C). Events altering the coding sequence of \textit{FOXD3} were seen in both HGB-03 (inter-chromosomal rearrangement) and HGB-04 (deletion of multiple exons). Both HGB-02 and HGB-04 showed deletions involving acetylated elements within the first intron of \textit{MEF2C}, a TF gene that is a mutational target in DLBCL (22). The functional significance of these events is unclear, as \textit{MEF2C} expression in these cases was similar to that seen in other samples (Supplementary Fig S2C).

PEAR-ChIP identified other small-scale intra-chromosomal deletions and inversions of potential oncogenic significance, including a 120 kb balanced inversion encompassing several exons of \textit{ETV6} (Supplementary Fig. S1D) and a deletion encompassing several exons of \textit{EBF1}. \textit{EBF1} is a frequent mutational target in DLBCL (13), while both \textit{ETV6} (23) and \textit{EBF1} (24) undergo frequent inactivation in immature B-cell neoplasms.

Unlike DLBCL, inter-chromosomal rearrangements are rare in CLL/SLL. PEAR-ChIP analysis of three SLL biopsies did reveal a t(3;7) rearrangement in SLL-03 that disrupted one allele of the \textit{CREB5} gene. In SLL-01, we detected a large interstitial deletion of chromosome 14 that linked the \textit{IGH} \(\mu\) switch region to the first intron of \textit{ZFP36L1} (Supplementary Fig. S1E), a known recurrent lesion in CLL/SLL (25). We also
detected both complete and incomplete (DJ only) immunoglobulin V(D)J rearrangements in all three SLL cases.

Enhancer tandem duplication as a mechanism of oncogene dysregulation in B-cell lymphoma

A particularly interesting class of events detected by PEAR-ChIP consisted of kilobase-scale tandem duplications affecting acetylated candidate enhancers. Such events could represent a mechanism of aberrant oncogene expression. One such duplication in HGB-01 involves a strongly acetylated candidate enhancer region upstream of the rho GTPase-activating gene \textit{TAGAP} (Fig. 2B). qRT-PCR data confirmed that HGB-01 has the highest \textit{TAGAP} expression level of 17 lymphomas evaluated, at 2.4 times the mean expression across samples (Fig. 2C), supporting the enhancer duplication as a gain-of-function event. Genetic polymorphisms within the \textit{TAGAP} locus have been linked to lymphocyte-mediated immune disorders, including rheumatoid arthritis, Crohn disease, and celiac disease, suggesting a role in lymphocyte regulation or proliferation, but, to our knowledge, \textit{TAGAP} has not been previously implicated in lymphoma.

Another notable tandem duplication event was identified upstream of the inducible nitric oxide synthase gene \textit{NOS2} in the PMBL specimen HGB-04 (Supplementary Fig. S1F). The duplicated sequence overlaps a 9 kb region that contains an interferon-responsive enhancer (26). Because interferon-stimulated gene expression is commonly mediated by IRF family TFs, we examined ENCODE ChIP-Seq data from interferon-\(\gamma\)-treated K562 cells, and noted a strong peak of IRF1 binding within the duplicated region. These findings suggest that the HGB-04 event duplicates a \textit{bona fide} enhancer. \textit{NOS2} expression has been associated with a poor prognosis in classical Hodgkin lymphoma, which shows strong genetic and phenotypic overlaps with PMBL (27). HGB-04 exhibits higher \textit{NOS2} expression than the average across our panel, but is not an outlier in this regard (Supplementary Fig. S2C).
Genomic duplication targets one of several BCL6-interacting super-enhancers

PEAR-ChIP analysis of PMBL case HGB-04 identified a 43 kb tandem duplication event encompassing a cluster of acetylated elements upstream of BCL6 (Fig. 3A), another critical lymphoma oncogene and frequent rearrangement target. To understand whether this region might represent a novel BCL6 enhancer, we performed a “super-enhancer” analysis (28) on H3K27ac data from our 29 B cell lines and primary samples. This highlighted a known BCL6-interacting enhancer region (6,16,17) 150-250 kb upstream of BCL6 (SE1), which overlaps the alternate breakpoint region, a recurrent site of BCL6 locus rearrangements (15). It also revealed a second super-enhancer overlapping the HGB-04 duplication at -350 kb (SE2), and a third such region at -500 kb (SE3). All three regions were strongly acetylated in normal centroblasts, GCB-DLBCL tumor biopsies, and most HGB cell lines, but lacked acetylation in non-germinal center B cells, MCL and SLL. The few HGB samples that lacked BCL6 super-enhancer activation included cases with evidence for a non-germinal center oncogenic program, such as our ABC-DLBCL sample HGB-01 and Oci-Ly-3, a well-characterized ABC-DLBCL cell line. We used chromosome conformation capture (3C) analysis of GCB-DLBCL lymphoma cell lines (Supplementary Fig. S3A) to confirm looping of elements from super-enhancer regions to the BCL6 promoter, consistent with roles in activating this oncogene. Intriguingly, our PMBL case, HGB-04, showed a distinct acetylation pattern across these regions, with only the duplicated enhancer region, SE2, showing strong acetylation. Despite lacking acetylation at most of the BCL6 enhancers seen in GCB-DLBCL, HGB-04 showed stronger BCL6 expression than 3 of 6 GCB-DLBCL cases, and all 5 ABC-DLBCL cases evaluated (Fig. 3B), suggesting that the tandem duplication of SE2 may drive BCL6 expression in this case.

Coordinated acetylation of BCL6 enhancers is driven by MEF2B binding and p300 recruitment
To better understand the basis for coordinated activation of BCL6 enhancers, we used ChIP-Seq in HGB cell lines to map MEF2B, a germinal center-specific TF known to drive BCL6 expression (29). We did not detect substantial MEF2B binding in the vicinity of the BCL6 promoter. Rather, we observed strong, focal MEF2B binding at acetylated elements within BCL6 super-enhancer regions, including four sites in SE1, one in SE2, and two in SE3 (Fig. 3C). These patterns were reproducible by two distinct MEF2B antibodies, and in five cell lines (Supplementary Fig. S3B-C). Since other MEF2 family proteins activate gene expression through recruitment of the p300 acetyltransferase to distal enhancers, we performed ChIP-Seq for p300. We observed a high degree of correlation between MEF2B and p300 binding at distal sites across the BCL6 locus (Fig. 3C), and genome-wide (Supplementary Fig. S4A). In contrast, p300 binding showed little correspondence to the distribution of three other B-cell TFs that we mapped (PU.1, Pax5, and Bach2). Genome-wide de novo motif analysis of all MEF2B ChIP-Seq datasets revealed a top-ranked motif similar to that published for other MEF2 family factors, and a MEF2-like motif represented the fourth most significantly enriched motif in p300 ChIP-seq peaks (Supplementary Fig. S4B-C). Intriguingly, sites within SE1 that were bound by other TFs, but not by MEF2B, showed minimal p300 binding, and retained acetylation in non-germinal center phenotype populations (Fig. 3A and 3C).

To test whether MEF2B expression was sufficient to drive BCL6 enhancer acetylation, we used an inducible lentiviral vector to express HA-tagged MEF2B in two MCL cell lines, which show low basal expression of BCL6 transcripts (Fig. 3D) and MEF2B protein (Supplementary Fig. S3C), as well as two HGB lines. Induction of MEF2B expression resulted in an increase in BCL6 expression in all cell lines, although the relative increase was greater in MCL lines (Fig. 3D). ChIP-Seq analysis in the Jeko-1 MCL line revealed a specific increase in H3K27ac at MEF2B binding sites in MEF2B transgene-expressing cells, but not at intervening elements within the BCL6 super-enhancers (Fig. 3E). These data support a direct role for MEF2B in the activation of enhancers that drive BCL6 expression in human lymphomas.
Native MYC locus enhancers show lymphoma subtype-specific activity

The MYC gene lies within a nearly 4-megabase locus devoid of other spliced protein-coding genes. This locus contains multiple enhancers that drive MYC expression in specific lineages and cancer types, including an immature T cell-specific enhancer (30) and a myeloid cell-specific enhancer (3), which reside 1.4 and 1.8 Mb downstream of MYC, respectively. Tissue-specific candidate MYC enhancers have also been identified for breast, colon, and prostate carcinomas (31). Although MYC enhancers from other cancers showed little acetylation in our B-cell lymphoma datasets, distinct patterns of acetylation identified other enhancer-like elements in the MYC locus (Fig 4A-C). Elements located between 235-535 kb downstream of MYC were frequently acetylated in HGB biopsies and cell lines (Fig. 4A,C), as well as in normal germinal center B cells (centroblasts). Elements in this region are syntenic to enhancers that interact with RNA polymerase II at the MYC promoter in mouse activated B cells (6,32), suggesting that they may represent human MYC enhancers. We used 3C in HGB cell lines to demonstrate that the MYC promoter physically interacts with candidate enhancers in this region (hereafter “3’ MYC-interacting region”), but not with the myeloid or T cell enhancers (Fig. 4A and data not shown). Intriguingly, the 3’ MYC-interacting region is targeted by recurrent focal somatic amplifications in plasma cell myeloma (33), further supporting a significant oncogenic role for this region in B-cell malignancies.

Although candidate enhancers in the 3’ MYC-interacting region were strongly acetylated in most HGB samples, they lacked acetylation in HGB biopsies and cell lines with genomic rearrangement of the MYC locus (Fig. 4C). Absence of acetylation in these cases appears to be biallelic, and independent of the position of the MYC locus breakpoint. If enhancers in this region are responsible for driving MYC expression, they would presumably be dispensable in the setting of translocations that bring strong heterologous enhancers into proximity with MYC. Their lack of acetylation in MYC-rearranged tumors could reflect negative-feedback mechanisms that suppress acetylation in the setting of high MYC...
expression. In support of this hypothesis, lentiviral overexpression of MYC in a non-rearranged HGB cell line caused a significant reduction in candidate enhancer acetylation within the 3’ MYC-interacting region (Supplementary Fig. 5A-B).

In contrast to HGB samples, most elements in the 3’ MYC-interacting region lacked acetylation in our MCL and SLL datasets, and in non-germinal center B cells from peripheral blood (PBL) (Fig. 4B-C). Instead, these populations showed specific acetylation of a cluster of candidate enhancers 400-600 kb upstream of MYC (hereafter “5’ MYC-interacting region”) that interact with the MYC promoter by 3C in MCL and SLL biopsies. Notably, the 5’ MYC-interacting region corresponds to a site of recurrent amplification in CLL/SLL (34), and contains regulatory elements that have previously been shown to interact with the MYC promoter and activate MYC transcription in in vitro-transformed B-lymphoblastoid cell lines via binding of the Epstein-Barr virus oncoprotein EBNA2 (35), demonstrating that elements within this region can function as bona fide MYC enhancers.

**Polymorphisms linked to hereditary risk for specific lymphomas are associated with MYC locus enhancers that show subtype-specific acetylation**

Genome-wide association studies (GWAS) have identified single-nucleotide polymorphisms (SNPs) in a small number of loci that correlate with altered hereditary risk for specific lymphoma types. Remarkably, we note that the 3’ MYC-interacting region, which shows centroblast and DLBCL/PMBL-specific enhancer acetylation, contains polymorphisms linked to hereditary risk for DLBCL (one of 4 known loci genome-wide) (36) and Hodgkin lymphoma (one of 6 known loci genome-wide) (37). Furthermore, the 5’ MYC-interacting region, which shows SLL and MCL-specific enhancer acetylation, contains a polymorphism linked to hereditary risk for CLL/SLL (one of 22 known loci genome-wide) (38). Intergenic polymorphisms predicted as causal for disease risk are frequently located within the central TF binding
region of enhancers, often adjacent to or directly altering TF binding motifs (39,40), suggesting that a similar mechanism may occur in lymphoma. Indeed, for each lymphoma type, we could identify at least one lead or strongly linked SNP ($r^2 > 0.9$) in the MYC locus that coincides precisely with a DNase hypersensitive site in an enhancer with subtype-specific acetylation (Supplementary Fig S5C-E). Further analysis revealed that the implicated SNPs alter or are adjacent to a high-confidence motif for a lymphoid TF. We note that rs7826019, one of the two enhancer-associated Hodgkin lymphoma SNPs that we highlighted, has been previously suggested as a likely functional SNP for this GWAS locus (41). The surrounding enhancer is acetylated in several HGB cases, including a biopsy of PBML, a disease that shares many common biological features with Hodgkin lymphoma (21). Taken together, our findings suggest that alternate regulatory elements located hundreds of kilobases upstream and downstream of the MYC gene are acetylated at distinct B cell developmental stages, and play oncogenic roles in specific lymphoma types.

**A t(3;8) translocation is characterized by MYC recruitment of BCL6 enhancers**

Identification of candidate enhancers in the BCL6 and MYC loci allowed us investigate aberrant enhancer-promoter relationships in the setting of the t(3;8)(q27;q24) rearrangement that directly links the MYC and BCL6 loci in the GCB-DLBCL sample HGB-07. HGB-07 showed the highest MYC expression by qRT-PCR of any of our lymphoma samples (Fig. 2C), but BCL6 expression in HGB-07 was the lowest of any GCB-DLBCL sample, although higher than in all ABC-DLBCL samples (Fig. 3B), suggesting that MYC, but not BCL6, was activated by this rearrangement. Interestingly, the BCL6 and MYC locus breakpoints in HGB-07 occur directly between each gene and its native enhancer regions, resulting in an “enhancer swap” (Fig. 5A-B). The 3’ MYC-interacting region in HGB-07 lacked acetylation, similar to the findings in lymphomas with known heterologous activating rearrangements of MYC. In contrast, the BCL6 super-enhancers in HGB-07 case were broadly acetylated, suggesting that these could function to activate MYC on the
rearranged allele. This hypothesis was supported by comparison of 3C data from HGB-07 to data from HGB-06, a lymphoma that lacked a MYC rearrangement, but showed strong BCL6 and moderate MYC expression by qRT-PCR and IHC. In both cases, we detected the expected interactions between the BCL6 and MYC promoters and candidate enhancers in their native locus (Fig. 5C-D). We then used the same enhancer primers to measure interactions with the opposite gene promoter. We detected strong interactions between the MYC promoter and elements within the BCL6 SE1 and SE2 regions in HGB-07, but no such interactions in the non-rearranged tumor, HGB-06. In contrast, we did not see significant interaction between the 3' MYC-interacting region and the BCL6 promoter in either case. Our findings strongly suggest that the oncogenic effect of this t(3;8) rearrangement is heterologous activation of MYC by BCL6 locus enhancers (see model in Fig. 5E).

**Discussion**

We have leveraged chromatin profiling and a novel analytic approach to identify genomic rearrangements associated with active enhancers, revealing both known “enhancer hijacking” events as well as novel enhancer duplications of likely oncogenic significance in lymphoma. Our focused investigation of two recurrently altered loci, MYC and BCL6, in the presence and absence of rearrangements and across multiple lymphoma subtypes, provided novel insights regarding the role of native and rearranged enhancers in controlling these critical oncogenes. Understanding such mechanisms may be of particular clinical significance, given the ongoing development of drugs that target enhancer function (42). Moreover, we believe that the efficient PEAR-ChIP approach described here expands future possibilities for identification of enhancer-associated rearrangements in research or clinical settings.

PEAR-ChIP efficiently detected genomic rearrangements in lymphoma samples, despite the fact that our datasets cover only a small fraction of the genome at high sequencing depth. Since many oncogene
rearrangements drive lymphoma by juxtaposing powerful enhancers with target oncogenes, it is not surprising that many such events should occur sufficiently close to strong enhancers to allow for breakpoint detection after enrichment for acetylated chromatin. Additionally, recent studies have demonstrated that AID, an important driver of DNA damage and genomic rearrangements in lymphomagenesis, is specifically targeted to strongly acetylated regions of the genome, which include both the immunoglobulin switch regions and a subset of strongly active enhancers genome-wide (6,7). By providing deep coverage of acetylated regions, PEAR-ChIP therefore focuses specifically on regions of the genome likely to harbor aberrant AID-induced rearrangements. Further studies are needed to determine the value of PEAR-ChIP analysis in cancer types other than lymphoma. One potentially fruitful strategy could be to complement the enhancer activation profiling and focused high-resolution breakpoint detection of PEAR-ChIP with a low-resolution, genome-wide approach such as long-insert mate-pair sequencing for efficient genome-wide correlation of enhancer activity and genomic rearrangements.

Our findings also provide novel insight regarding the activity of lymphoma subtype-specific candidate enhancers in the native loci of the oncogenes \textit{MYC} and \textit{BCL6}. Such native locus enhancers may drive oncogene expression in lymphomas without rearrangements, and may be further activated by focal duplication or amplification events. Additionally, our analysis suggests that germline polymorphisms in the \textit{MYC} locus linked to altered risk for specific lymphoma types may directly affect enhancers that are selectively active in the corresponding lymphomas. Additional work is needed to demonstrate whether the specific enhancer-associated SNPs highlighted here are truly causal for altered lymphoma risk, the mechanisms by which they act, and the implications for lymphoma prevention and treatment. Importantly, we found that a master TF for \textit{BCL6} expression, MEF2B, drives acetylation of specific individual elements within multiple ‘super-enhancers’ that converge on the \textit{BCL6} promoter. Understanding cross-talk and synergy between individual TF units in this array could be an important
area for future study, and lies at the crux of more general questions regarding the super-enhancer concept (43).

Finally, our investigation sheds light on the function of a t(3;8) **BCL6-MYC** rearrangement, a recurrent event in B-cell lymphomas (44). Among DLBCL cases, **BCL6** rearrangements are most often seen in ABC-DLBCL (45), and often involve partner loci with strong activating elements, such as **IGH**, **IGK**, and **IGL**, which are thought to activate expression of **BCL6** in the absence of an intact germinal center program. In contrast, our data shows that the t(3;8) event in HGB-07, a case of GCB-DLBCL, is unlikely to be a **BCL6** activating event, but rather results in **BCL6** enhancers interacting with, and likely activating, **MYC**. Thus, the **BCL6** locus may serve as either the recipient or the donor of activating regulatory elements in distinct rearrangements. To our knowledge, this is the first example of any oncogene locus capable of playing such a dual role. This finding has important implications for defining a group of aggressive B-cell lymphomas bearing simultaneous activating translocations of **MYC** plus one or both of the oncogenes **BCL2** and **BCL6**, referred to as “double hit lymphomas” (DHL) or “triple hit lymphomas” (THL) (46). These cases have been associated with a poor prognosis, and are frequently treated with an intensified initial chemotherapeutic regimen. In some series, the presence of a t(3;8) **BCL6-MYC** rearrangement, or positive results on break-apart FISH-studies for both the **BCL6** and **MYC** loci (without identifying partner loci) have been considered sufficient to classify a case as DHL. Our data challenges this assumption by demonstrating that the t(3;8) rearrangement in HGB-07 is unlikely to be a **BCL6**-activating event, but rather represents **MYC** activation by **BCL6** locus enhancers, similar to other “single-hit” **MYC**-activating rearrangements. We would suggest that lymphomas bearing t(3;8) **BCL6-MYC** rearrangements should be distinguished from cases with separate activating translocations of **BCL6** and **MYC** to different partner loci, and propose the term “pseudo double-hit rearrangement” for events of the type seen in HGB-07. More generally, given the structural and functional diversity of **BCL6** locus rearrangements, we would
caution against the assumption that all FISH-detected BCL6 rearrangements carry biologically similar implications for the purposes of research or clinical diagnostic practice.

**Materials and Methods**

**Cell lines and lymphoma samples**

Lymphoma cell lines were generous gifts from Dr. Mark Minden at the Ontario Cancer Institute (Oci-Ly-1 and Oci-Ly-7, acquired in 2010), were purchased from ATCC (Pfeiffer and Toledo, acquired in 2013) or were obtained from the Broad-Novartis Cancer Cell Line Encyclopedia (all others, acquired between 2012 and 2014) and were grown in IMDM + 20% FCS (Oci-Ly-1 and Oci-Ly-7) or RPMI + 10-20% FCS (all others). Pfeiffer and Toledo were validated at ATCC by short tandem repeat profiling, while identity of all other lines was validated by Sanger sequencing of unique polymorphisms at the time of acquisition and frozen stock generation in our laboratory. With the exception of lentivirus experiments, all lines were grown for <10 passages in our laboratory between validation sequencing and crosslinking for ChIP-Seq. ChIP-Seq profiling of normal B cell populations was described previously (40). Frozen excess surgical tissue from human lymphomas was obtained from the Massachusetts General Hospital Pathology Service under DF/HCC protocol 13-594. Informed consent was obtained from each subject. Frozen sections and paraffin IHC were reviewed, and blocks with >80% lymphoma cells were used. IHC for CD10, BCL6, IRF4, and MYC were performed on paraffin sections of lymphoma biopsies according to protocols validated for clinical diagnostic use by the MGH Pathology Service, and DLBCL cases were preliminarily classified as GCB- or non-GCB-DLBCL by the Hans algorithm (47). Interphase FISH analysis for MYC rearrangement was performed with the Vysis LSI MYC Dual Color, Break Apart Rearrangement Probe (Abbott Molecular).

ChIP-Seq
For histone modification and TF ChIP-Seq in cell lines, 10-20 million cells were crosslinked in growth media + 1% formaldehyde for 10 min. at 37°C, quenched for 5 min. with 125 mM glycine, washed twice in cold PBS with protease inhibitors, and stored at -80°C. For primary lymphoma samples, tumor cellularity from the frozen block was confirmed by H&E frozen section by a board-certified hematopathologist (RR), and the block was trimmed as needed. 25 micron sections were then cut to a total of ~50 mg of tissue for each ChIP-Seq chromatin prep. Frozen sections were resuspended and dissociated in PBS + PI + 10 mM sodium butyrate, formaldehyde was added to 1%, and crosslinking, quenching and washing were performed as above. Chromatin immunoprecipitation and sequencing library preparation were performed by standard methods, with details provided in the Supplementary Methods.

Paired-end sequencing of H3K27ac ChIP and an un-enriched chromatin control library was performed on a HiSeq 2500 or NextSeq for all primary lymphoma samples and for 8 cell lines, with read lengths of 36 bp + 25 bp or 38 bp + 38 bp. Based on analysis of mapped read-pairs, paired-end libraries contained an average fragment size of 303 bp (range 252-332 bp) with 95% of fragments at least 147-225 bp and at most 398-498 bp in length. Single-end sequencing was used for other samples. Single- and paired-end reads were aligned to hg19 using BWA-ALN and filtered to remove PCR duplicates and reads mapping to >2 sites genome-wide. ChIP-Seq tracks were generated with ‘igvtools count’ and visualized with IGV.

Super-enhancer analysis of H3K27ac ChIP-Seq was performed with MACS and ROSE as described (28). TF and p300 ChIP-seq peak calling were performed with HOMER, using the “factor” style. De novo motif analysis of TF and p300 peaks was performed using HOMER. ChIP datasets are available through Gene Expression Omnibus accession GSE69558, and dbGaP accession phs000939.v1.p1.

PEAR-ChIP Rearrangement detection

dRanger and BreakPointer (10) were used to identify rearrangements in paired-end H3K27ac alignment files. Customized filtering criteria are detailed in the Supplementary Methods.
RT-PCR, qRT-PCR and ChIP-PCR

25 micron sections of frozen tumor tissue were used for RNA extraction via sequential Trizol and column purification with DNase digestion, followed by reverse transcription. RT-PCR primers for confirmation of the NCOA3-PDCD1LG2 fusion transcript consisted of a forward primer within NCOA3 exon 1, and a reverse primer located after the first splice junction of PDCD1LG2. To support our classification of biological subtype for high-grade lymphomas, we designed qPCR primers for signature genes identified as significantly up-regulated in PMBL versus DLBCL in two expression microarray studies (21,48) and for selected GCB-DLBCL and ABC-DLBCL signature genes, as well as all five housekeeping genes, from a validated assay for lymphoma expression classification (49). Syber Green qRT-PCR was used to measure the expression of rearrangement target genes, signature genes, and housekeeping genes, with RPLPO used as a reference gene on all plates. Relative quantity and 95% confidence intervals were calculated for each gene by the delta-delta Ct method and ABI 7500 software. All relative expression values for each sample were then divided by a normalization factor, calculated as the geometric mean of the quantities of each of the five housekeeping genes in that sample relative to sample HGB-01. For cell line experiments, SYBER Green qRT-PCR was used to measure BCL6 expression by the relative standard curve method with normalization to RPLP0. The relative change in MYC locus promoter and enhancer acetylation were quantified by SYBER green qPCR of ChIP DNA, with normalization to input chromatin and primers recognizing control enhancers adjacent to the CD79A and PTPRC (CD45) genes. All primer sequences are available on request.

Single nucleotide polymorphism linkage and motif analysis

MYC locus polymorphisms linked to germline risk for development of DLBCL, Hodgkin lymphoma, and chronic lymphocytic leukemia were obtained from meta-analyses of genome-wide association studies (GWAS) conducted in populations of British, European-American, and other Western European origin
We used the 1000 Genomes Project Browser to identify all SNPs in strong linkage disequilibrium \((r^2 > 0.8)\) to identified GWAS index SNPs in both the GBR and CEU population subgroups. Index SNPs and all linked SNPs were queried for overlap with ENCODE uniform DNase hypersensitivity (DNAse HS) peaks for 10 B-lymphoblastoid cell lines and 2 other B cell populations. HOMER findMotifs was used to identify and score known TF binding motifs in the presence of the reference or variant allele for each SNP in a DNase HS site.

**Chromosome conformation capture**

3C was performed on 10 million cultured cells or 20-40 mg of 25 micron-sectioned frozen tissue according to published protocols (50). Taqman probe and primer sets for the \(BCL6\) locus were adapted from previous reports (16). Probe and primer sets for EcoRI 3C at the \(MYC\) locus were designed with Primer3 for mutual compatibility with \(BCL6\) 3C primers. All comparisons reflected equal input DNA quantity as determined by SYBER green qPCR. Taqman qPCR was performed in 2-3 replicates for all sites, and a Ct value of 40 was assigned for all reactions with \(Ct \geq 40\). Values are expressed as relative quantity \([2^{-(Ct_{test} - Ctprom})]\) in comparison to control primers spanning the EcoRI site at the \(MYC\) promoter, and error bars reflect the standard error of the mean for replicates. HindIII-based 3C was performed to evaluate the T-cell-specific \(MYC\) enhancer with published probes and primers (30). Probe and primer sequences are available on request.

**Lentiviral constructs**

An open reading frame encoding \(MEF2B\) (CCDS 46024.1) with a c-terminal HA tag was cloned into pINDUCER-20, and \(MYC\) (CCDS 6359.2, amino acids 16-454) was cloned into pLX-304. Lentivirus was produced in HEK-293T cells and subject to 0.45-µm filtration. Cell lines were transduced with lentiviral supernatants by spinfection for 90 minutes in the presence of polybrene. After 48 hour recovery,
transduced cells were selected by blastocidin (5 days, pLX-304), or G418 (10+ days, pINDUCER-20). pINDUCER-20 expression was induced with 100 ng/mL doxycycline 48 hours prior to cell harvest.

Western Blot

Western blots were performed by standard protocols with the following antibodies: MEF2B (ab33540, Abcam and HPA004734, Atlas), MEF2C (D80C1, Cell Signalling), and TBP (1TBP18, Abcam)

Acknowledgments

The authors would like to thank all clinicians and staff who facilitated identification and consenting of patients, including J. Abramson, R. Takvorian, J. Barnes, Y. Chen, T. Spitzer, B. McGree, A. Crawford, J. Ramirez, N. Birrer, and H. Martinson, as well as C. Desilus for technical assistance with FISH assays. We thanks Nancy L. Harris for thoughtful feedback on the manuscript. R. Ryan, A. Sohani, and E. Hochberg gratefully acknowledge funding support from an anonymous foundation.

References


**Figure Legends**

**Figure 1:** Detection of rearrangements involving the *CCND1* and *MYC* loci by PEAR-ChIP.  
*Left* - Schematic depiction of a rearrangement between two chromosomes (red and green) with the breakpoint located in chromatin marked by H3K27ac (purple triangles). ChiP-Seq leads to isolation of H3K27ac-associated DNA, including enhancer-associated breakpoints. *Right* - Sequencing and alignment of ChiP DNA from case MCL-01 identifies read pairs at the ends of fragments containing the (11;14) breakpoint (orange reads) and a physiological *IGH* VDJ recombination (red reads).  
*Tracks* showing H3K27ac signal in normal B cells (salmon), MCL cell lines (maroon) and MCL biopsies (dark purple) at the *IGH* and *CCND1* loci. *IGH-CCND1* rearrangement breakpoints detected by PEAR-ChIP are marked with black arrowheads. Also shown are breakpoints corresponding to intra-chromosomal deletions (paired open arrowheads) or large-scale rearrangements (single open arrowheads) affecting the *CCND1* 3’UTR.  
*Tracks* showing H3K27ac tracks and location of PEAR-ChIP-detected large-scale rearrangements (black arrows) involving the *MYC* locus.

**Figure 2:** Genome-wide rearrangement detection by PEAR-ChIP.  
*Tracks* summarizing inter-chromosomal (dark shades) and intra-chromosomal (light shades) genomic rearrangements detected by
H3K27ac PEAR-ChIP in 8 lymphoma cell lines (top) and 14 patient biopsies (bottom). Gene labels mark selected loci of interest. B. H3K27ac tracks and location of large-scale rearrangements (black arrows) detected by PEAR-ChIP at known oncogene (BCL2 and PDCD1LG2) and tumor suppressor gene (SGK1 and CIITA) loci, as well as a tandem duplication encompassing candidate enhancers upstream of TAGAP. Red bars link divergent read pairs spanning the tandem duplication. C. Normalized RNA expression (RQ = relative quantity) for genes affected by PEAR-ChIP-detected genomic lesions in HGB biopsies. Numbers at bottom denote the HGB sample name (e.g. “1” = HGB-01). Symbols indicate samples that contain a genomic lesion proximal to the measured gene (asterisk: inter-chromosomal rearrangement predicted to activate gene expression; minus sign: rearrangement predicted to inactivate gene expression; plus sign: enhancer tandem duplication). The pound sign denotes a FISH-detected rearrangement between the MYC and BCL6 loci. Samples are color-coded by gene expression subtype as follows: green = GCB-DLBCL, red = ABC-DLBCL, blue = PMBL, gray = unclassified. Solid bars denote samples studied by PEAR-ChIP.

**Figure 3:** Acetylation and rearrangement of BCL6 locus enhancers. A. H3K27ac ChIP-Seq tracks across the BCL6 locus in 29 B cell populations. Green bars at bottom indicate the median positions of detected super-enhancer regions. Black brackets indicate published breakpoint cluster regions. Read-pairs spanning the tandem duplication in HGB-04 are linked by red bars at bottom. The legend at left indicates the super-enhancers called in each population, as well as populations classified as HGB (red = ABC-DLBCL, green = GCB-DLBCL, blue = PMBL, black = HGB cell line). MEF2B-positive (black arrows) and selected MEF2B-negative TF binding sites (open arrows) are indicated, and correspond to positions indicated in (C). ChIP-seq coverage range is 0-5 fpm (fragments per million mapped fragments) for all tracks. B. Normalized RNA expression of BCL6 in HGB biopsies. Samples are numbered and color-coded by subtype as in Figure 2C. Solid boxes indicate samples evaluated by PEAR-ChIP. Symbols indicate samples that contain a genomic lesion proximal to the measured gene (plus sign: enhancer tandem duplication, pound sign: FISH-detected rearrangement between the MYC and BCL6 loci). C. ChIP-Seq tracks for p300, H3K27ac, and
three TFs in two HGB cell lines at the BCL6 promoter and super-enhancer regions. MEF2B-positive (black arrows) and selected MEF2B-negative TF binding sites (open arrows) are indicated and correspond to positions indicated in (A). ChIP-Seq coverage range is 0-15 fpm for H3K27ac. D. Relative BCL6 expression in MCL and HGB cell lines stably transduced with pINDUCER-20 bearing a GFP or MEF2B-HA transgene, and harvested 48 hours after induction with 100 ng/ml doxycycline. E. ChIP-seq tracks for H3K27ac and HA-tag in Jeko-1 cells with induced expression of GFP or MEF2B-HA as in figure 4b. Genomic positions and arrows are identical to (C). ChIP-seq coverage range is 0-2.5 fpm for H3K27ac.

Figure 4: Enhancer acetylation at native and rearranged MYC loci in B-cell lymphoma. A. Interaction of candidate MYC locus enhancer regions with the MYC promoter by chromatin conformation capture (3C) in HGB cell lines (top), and corresponding H3K27ac ChIP-Seq tracks (bottom). Red bar at bottom indicates 3’ MYC-interacting region detailed in (C). Arrows indicate the positions of previously reported MYC enhancers in T cell leukemia (black arrow) (30) and myeloid leukemia (open arrow) (3). ChIP-seq coverage range is 0-10 fpm. B. Interaction of candidate MYC locus enhancer regions with the MYC promoter by chromatin conformation capture (3C) in MCL and SLL biopsies (top), and corresponding H3K27ac ChIP-Seq tracks (bottom). Red bar at bottom indicates 5’ MYC-interacting region detailed in (C). ChIP-seq coverage range is 0-5 fpm. C. Detail of H3K27ac ChIP-seq tracks at MYC-interacting regions in B cell samples. Legend at center indicates lymphoma type (for HGB biopsies red = ABC-DLBCL, green = GCB-DLBCL, blue = PMBL) or normal B population, presence or absence of a MYC rearrangement by fluorescence in situ hybridization (“MYC FISH”, orange: rearrangement, gray: not detected, white: not evaluated), and MYC rearrangement detection by PEAR-ChIP (“PC MYC-R”). Triangles at bottom indicate position and lymphoma subtype associations of single-nucleotide polymorphisms linked to hereditary risk for lymphoma in published GWAS studies. ChIP-seq coverage range is 0-5 fpm for all tracks.
Figure 5: A t(3;8)(q27;q24) rearrangement leads to MYC activation by BCL6 enhancers. A. H3K27ac ChIP-Seq tracks at the BCL6 locus for HGB-06 and HGB-07. The MYC promoter region is also shown for HGB-07, and a dashed black line connects the t(3;8) rearrangement breakpoints. Solid black (HGB-06) or red (HGB-07) arrows depict looping interactions on the native chromosome 3 as determined by 3C. Dashed red arrows depict looping interactions between BCL6 enhancers and the MYC promoter in HGB-07, as determined by 3C. B. H3K27ac ChIP-Seq tracks at the MYC locus for HGB-06 and HGB-07. The BCL6 promoter region is also shown for HGB-07, and a dashed black line connects the t(3;8) rearrangement breakpoints in HGB-07. Solid black (HGB-06) or red (HGB-07) arrows depict looping interactions on the native chromosome 8 as determined by 3C. C. 3C data for interactions between BCL6 locus enhancers and the BCL6 promoter (top) or MYC promoter (bottom) for HGB-06 and HGB-07. “B” indicates the position of the breakpoint junction on the der(8)t(3;8) in HGB-07. D. 3C data for interactions between MYC locus distal elements and the MYC promoter (top) or BCL6 promoter (bottom) for HGB-06 and HGB-07. “B” indicates the position of the breakpoint junction on the der(3)t(3;8) in HGB-07. E. Schematic models of MYC promoter interactions with native and heterologous enhancers in B cell lymphomas.
Figure 1

A. Jeko-1 (MCL)

B. Oci-Ly-1 (HGB)

C. DOHH-2 (HGB)

D. HGB-05

Figure 1: Mantle cell lymphoma

Figure 1A: Mantle cell lymphoma

Figure 1B: Mantle cell lymphoma

Figure 1C: Mantle cell lymphoma
A. 

Figure 2

HGB-01

Tandem duplication

TAGAP

HGB-05

HGB-04

HGB-01

C.
Figure 3

A. 

B. 

C. 

D. 

E.
**Figure 4**

A. 0.003  
Karpas-422 (HGB line)  
Oci-Ly-7 (HGB line)  
MYC  
PVT1  
GSDMC  
FAM49B  
500 kb  

B. 0.0015  
SLL-01  
MCL-03  
MYC  
PVT1  
GSDMC  
FAM49B  

C.  

**5' MYC-interacting region**  
chr8:128,000,000-128,330,000  

**3' MYC-interacting region**  
chr8:128,983,865-129,283,865  

Centroblast  
Karpas-422  
Pfeiffer  
Su-DHL-6  
HGB-01  
HGB-02  
HGB-03  
HGB-04  
HGB-06  
HGB-05  
HGB-07  
DOHH-2  
Oci-Ly-1  
Oci-Ly-7  
Jeko-1  
Rec-1  
Mino  
Granta-519  
MCL-01  
MCL-02  
MCL-03  
MCL-04  
SLL-01  
SLL-02  
SLL-03  
PBL B cells  
RP11-255B23.4  
rs2456449 (CLL/SLL risk)  

Karpas-422  
Oci-Ly-7  
SLL-01  
MCL-03  

0.0015  

rs13255292 (DLBCL risk)  
rs2019960 (HL risk)  
rs4733601 (DLBCL risk)  

rs13255292 (DLBCL risk)  
rs2019960 (HL risk)  
rs4733601 (DLBCL risk)  

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Figure 5

A. HGB-06

B. HGB-07

C. 0.012
   0.008
   0.004
   0.002
   0.001
   0.000
   BCL6
   MYC
   HGB-06
   HGB-07

D. 0.002
   0.001
   0.000
   BCL6
   MYC
   HGB-06
   HGB-07

E. Native 5' MYC enhancers
   Small lymphocytic lymphoma
   Mantle cell lymphoma

Native 3' MYC enhancers
   Centroblasts
   DLBCL

BCL6 enhancers
Detection of Enhancer-Associated Rearrangements Reveals Mechanisms of Oncogene Dysregulation in B-cell Lymphoma

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Cancer Discovery  Published OnlineFirst July 30, 2015.

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