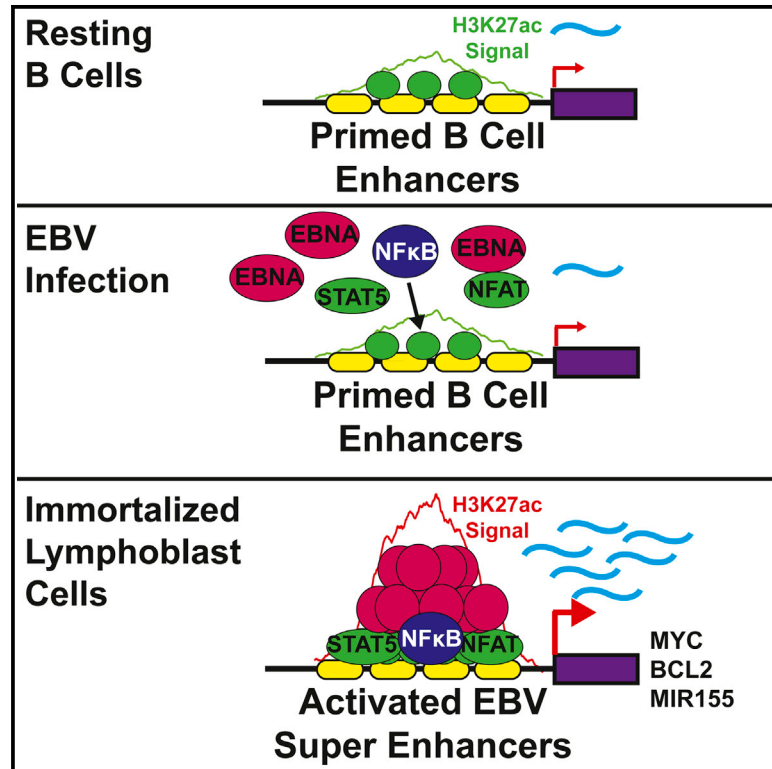


# Cell Host & Microbe

## Epstein-Barr Virus Oncoprotein Super-enhancers Control B Cell Growth

### Graphical Abstract



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### In Brief

Super-enhancers are gene-regulatory elements that determine cell transcription, development, phenotype, and oncogenesis but have not been implicated in host-pathogen interactions. Zhou and colleagues find that Epstein-Barr virus transcription factors and virus-activated NF-κB subunits converge into super-enhancers in lymphoblastoid cells to govern key oncogene expression and cause continuous cell growth.

### Highlights

- EBV transcription factors and NF-κB subunits converge into EBV super-enhancers
- MYC and BCL2 expression is driven by EBV super-enhancers
- EBV super-enhancers are co-occupied by B cell transcription factors and cofactors
- EBV super-enhancers are sensitive to perturbations



# Epstein-Barr Virus Oncoprotein Super-enhancers Control B Cell Growth

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## SUMMARY

Super-enhancers are clusters of gene-regulatory sites bound by multiple transcription factors that govern cell transcription, development, phenotype, and oncogenesis. By examining Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs), we identified four EBV oncoproteins and five EBV-activated NF- $\kappa$ B subunits co-occupying ~1,800 enhancer sites. Of these, 187 had markedly higher and broader histone H3K27ac signals, characteristic of super-enhancers, and were designated “EBV super-enhancers.” EBV super-enhancer-associated genes included the *MYC* and *BCL2* oncogenes, which enable LCL proliferation and survival. EBV super-enhancers were enriched for B cell transcription factor motifs and had high co-occupancy of STAT5 and NFAT transcription factors (TFs). EBV super-enhancer-associated genes were more highly expressed than other LCL genes. Disrupting EBV super-enhancers by the bromodomain inhibitor JQ1 or conditionally inactivating an EBV oncoprotein or NF- $\kappa$ B decreased *MYC* or *BCL2* expression and arrested LCL growth. These findings provide insight into mechanisms of EBV-induced lymphoproliferation and identify potential therapeutic interventions.

## INTRODUCTION

Epstein-Barr virus (EBV), the first human tumor virus, discovered 50 years ago in African Burkitt's lymphoma cells (Epstein et al., 1964), is causally associated with infectious mononucleosis, Burkitt's lymphoma, Hodgkin's lymphoma, HIV-related lymphomas, posttransplant lymphoproliferative diseases (PTLDs), nasopharyngeal carcinoma, and some gastric cancers (Longnecker et al., 2013; Young and Rickinson, 2004). In primary EBV infection, virus transits across the oropharyngeal epithelium to reach the B cell compartment. EBV converts primary B cells into activated blasts, which enable EBV to colonize the B cell compartment. Indeed, EBV-transformed lymphoblasts can be

seen transiently in patients with infectious mononucleosis, which is caused by primary EBV infection (Kurth et al., 2000). Although T- and NK-cell surveillance eventually contains lymphoblast proliferation, EBV latently infected B cells are the reservoir from which the virus establishes lifelong infection. With HIV infection, organ transplantation, or primary immunodeficiency, impaired control of EBV latently infected B cells leads to fatal lymphoproliferative diseases and lymphomas. In vitro, EBV transforms primary resting B lymphocytes (RBLs) to continuously proliferating lymphoblastoid cell lines (LCLs). LCLs express the same viral genes as some EBV lymphomas. These viral genes include six Epstein-Barr virus nuclear antigens (EBNAs), three latent membrane proteins (LMPs), and multiple microRNAs (Longnecker et al., 2013; Young and Rickinson, 2004). LCLs are therefore a useful model for studying EBV mediated B-lymphoid oncogenesis (Longnecker et al., 2013).

Reverse genetic studies indicate that viral oncoproteins EBNA2, EBNA1P, EBNA3A, EBNA3C, and LMP1 are each required for LCL growth and survival (Longnecker et al., 2013). EBNAs bind to virus and cell DNA through their interactions with cell DNA binding proteins. EBNA2 and EBNA1P are the first EBV genes expressed after B cell infection (Alfieri et al., 1991). EBNA2 mostly binds to DNA through the cell DNA binding protein RBPJ, and activates cell gene transcription, including *MYC*, the EBV cell surface receptor *CD21*, and *CD23* (Grossman et al., 1994; Henkel et al., 1994; Kaiser et al., 1999; Wang et al., 1987; Zhao et al., 2011b). EBNA2 binds to B cell enhancer sites ~428 kb and 525 kb upstream of *MYC* and loops to the *MYC* TSS to activate *MYC* transcription (Zhao et al., 2011b). The EBNA2 C-terminal acidic activation domain recruits basal and activation-related TFs, including Pol II, p300/CBP, TFIID, and TFIIF (Tong et al., 1995; Wang et al., 2000). EBNA1P coactivates with EBNA2 and derepresses transcription by removing NCoR and associated repressors from promoter DNA (Harada and Kieff, 1997; Portal et al., 2006, 2011, 2013). EBNA3A and EBNA3C repress *p16<sup>INK4A</sup>* and *p14<sup>ARF</sup>* expression, thereby preventing senescence and enabling continuous LCL growth (Maruo et al., 2011; Skalska et al., 2013). EBNA3A and EBNA3C also affect the expression of many host genes (Hertle et al., 2009; Zhao et al., 2011a). However, the growth-inhibiting effects of EBNA3A or EBNA3C deficiency can only be rescued by restoring EBNA3A or EBNA3C expression (Maruo et al., 2005, 2006), indicating that they both have unique nonredundant functions.

LMP1 constitutively activates NF- $\kappa$ B to promote growth and survival. NF- $\kappa$ Bs are dimeric TFs assembled from the RelA, RelB, cRel, p50, and p52 subunits. In resting B cells, NF- $\kappa$ B is tethered in the cytosol by I $\kappa$ B $\alpha$ . In LCLs, LMP1 signaling triggers I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B homo- or heterodimer nuclear translocation. Inducible overexpression of a nondegradable I $\kappa$ B $\alpha$  blocks NF- $\kappa$ B activity and causes LCL apoptosis (Cahir-McFarland et al., 2000, 2004). Conditional inactivation of EBNA2, EBNA3A, EBNA3C, or NF- $\kappa$ B identified cell genes regulated by these EBV oncoproteins. However, only a limited repertoire of cell genes are coregulated by EBNA2 or NF- $\kappa$ B (Cahir-McFarland et al., 2004; Hertle et al., 2009; Zhao et al., 2006, 2011a).

Super-enhancers are recently discovered enhancers with extraordinarily high and broad ChIP-seq signals for activation-related TFs, H3K27ac modification, bromodomain binding protein, BRD4, or mediator Med1 (Chapuy et al., 2013; Hnisz et al., 2013; Lovén et al., 2013; Parker et al., 2013; Whyte et al., 2013). Super-enhancers are principle determinants of cell identity and oncogenesis, although a super-enhancer role in host-pathogen interactions has not yet been reported. Super-enhancers are associated with genes critical for cell function, are co-occupied by multiple TFs in dense clusters, and are more sensitive to perturbation than typical enhancers (Chapuy et al., 2013; Hnisz et al., 2013; Lovén et al., 2013; Whyte et al., 2013). Super-enhancer formation can also be rapidly induced de novo upon cytokine stimulation accompanied by the decommitment of parental cell super-enhancers (Brown et al., 2014). BRD4 inhibition by bromodomain inhibitor JQ1 has significantly larger effects on super-enhancer-associated gene expression than on typical enhancer-associated gene expression (Hnisz et al., 2013; Lovén et al., 2013). In multiple myeloma (Lovén et al., 2013) and diffuse large B cell lymphomas (DLBCLs) (Chapuy et al., 2013), the *MYC* oncogene is controlled by super-enhancers.

We have now used EBNA2; EBNA3A; EBNA3C; NF- $\kappa$ B subunits RelA, RelB, cRel, p50, and p52; and ENCODE GM12878 LCL TF ChIP-seq data for integrated analyses of EBV super-enhancer effects on LCL growth. We were surprised to find all four oncogenic EBNA2s and all five NF- $\kappa$ B subunits co-occupying 187 sites that had extraordinarily high H3K27ac signals, indicative of super-enhancers. As characteristic of super-enhancers, EBV super-enhancers regulated key B cell growth and survival genes, and super-enhancer disruption by the BRD4 inhibitor JQ1, or by EBNA2 or NF- $\kappa$ B inactivation, inhibited EBV super-enhancer-associated gene expression and LCL growth.

## RESULTS

### EBNA2 Super-enhancers

Markedly elevated ChIP-seq signals of the B cell master TF SPI1/PU.1 distinguish B cell super-enhancers from typical enhancers (Whyte et al., 2013). Interestingly, we previously found that LCL EBNA2 sites were enriched for the SPI1 motif, and were frequently co-occupied by SPI1, suggesting possible EBNA2 incorporation into super-enhancers (Zhao et al., 2011b). EBNA2 ChIP-Seq signals were therefore tested for hallmarks of super-enhancer formation (Whyte et al., 2013). Model-based

analysis of ChIP-Seq (MACS) identified 42,251 EBNA2 sites with  $p < 10^{-5}$  (Zhang et al., 2008). Many EBNA2 sites were in broad clusters, characteristic of super-enhancers. EBNA2 sites within 12.5 kb windows were then merged into 16,133 EBNA2 clusters (Whyte et al., 2013) and ranked by their EBNA2 ChIP-Seq signal. Interestingly, 888 (5.5%) of the EBNA2 sites had ChIP-seq signals 23 times higher than typical EBNA2 sites and were >4 kb wide (see Table S1 available online; Figure 1A). These sites were therefore subsequently referred to as EBNA2 super-enhancers.

As expected, most EBNA2 super-enhancers were not near transcription start sites (TSSs) (Zhao et al., 2011b). We first assigned EBNA2 super-enhancers to their nearest genes (Whyte et al., 2013). Chromatin conformation capture (3C) followed by deep sequencing (Hi-C) captures long-range enhancer-promoter interactions and defines genome topological association domains (TADs) (Lieberman-Aiden et al., 2009). We tested our EBNA2 super-enhancer-associated gene assignments using high-resolution GM12878 LCL Hi-C data (Selvaraj et al., 2013). Approximately 86% of EBNA2 super-enhancers and their associated gene pairs occurred within the same Hi-C TAD, in agreement with super-enhancers and their targeted genes frequently co-occurring within CTCF/Cohesin domains (Dowen et al., 2014).

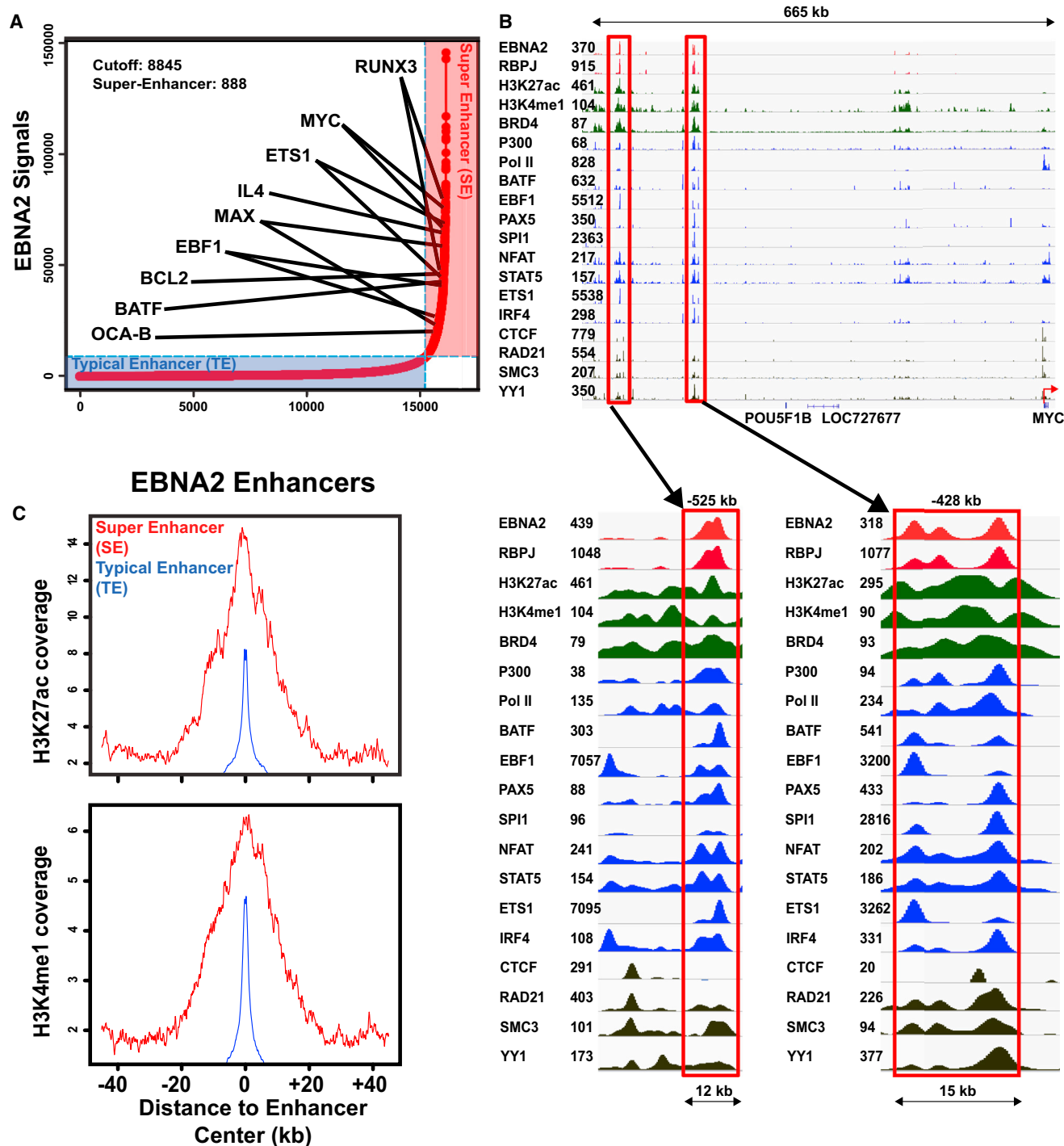
EBNA2 super-enhancers frequently localized near genes encoding relevant B cell TFs, including *MYC*, *MAX*, *EBF*, *RUNX3*, *ETS1*, and *BATF*, as well as the B cell-specific coactivator *OCAB* (Figure 1A). Many other cell TFs, including *BATF*, *EBF*, *ETS1*, *IRF4*, *SPI1*, *NFAT*, *STAT5*, and *PAX5*, co-occupied EBNA2 super-enhancer sites. *RBPJ* also had significant signals at these sites. Cell TFs involved in chromatin looping, such as *CTCF*, *SMC3*, and *RAD21*, were at adjacent sites (Figure 1B).

EBNA2 upregulation of *MYC* is essential for LCL growth (Fau-mont et al., 2009). EBNA2 super-enhancers at  $\sim$ –525 kb and –428 kb of the *MYC* TSS likely induce *MYC* expression (Alfieri et al., 1991; Kaiser et al., 1999; Zhao et al., 2011b) (Figure 1B). The –428 kb EBNA2 super-enhancer site loops to the *MYC* TSS in an EBNA2-dependent manner by fluorescence in situ hybridization (FISH) and 3C-qPCR (Zhao et al., 2011b). *MYC* heterodimerizes with *MAX* to activate *Cyclin D2* expression, promote cell cycle entry, and enable LCL DNA replication. Two EBNA2 super-enhancer sites also localized near *MAX* and likely affect *MAX* expression (Figure 1A). EBNA2 also upregulates *RUNX3* expression and represses *RUNX1* expression (Spender et al., 1999).

The 888 EBNA2 super-enhancer sites (SE in Figure 1C) had much higher H3K27ac and H3K4me1 signals than EBNA2 typical enhancer (TE) sites, indicative of a higher transcription activation state (Figure 1C).

### EBV Super-enhancers

EBNA2 and EBNA3C colocalize at *MYC* enhancers, with RelA and EBNA3A (Jiang et al., 2014; Portal et al., 2013; Zhao et al., 2011b, 2014). EBNA3A, RelB, cREL, p50, and p52 binding at EBNA2 *MYC* super-enhancers was evaluated. Surprisingly, all oncogenic EBNA2s and NF- $\kappa$ B subunits were at the *MYC* enhancer site (Figure 2A). We therefore searched for genome-wide co-occurrences of all EBNA2s and NF- $\kappa$ B subunits. In total, 1,771 sites had significant signals for all EBNA2s and NF- $\kappa$ B subunits, and were therefore designated as EBV enhancers.

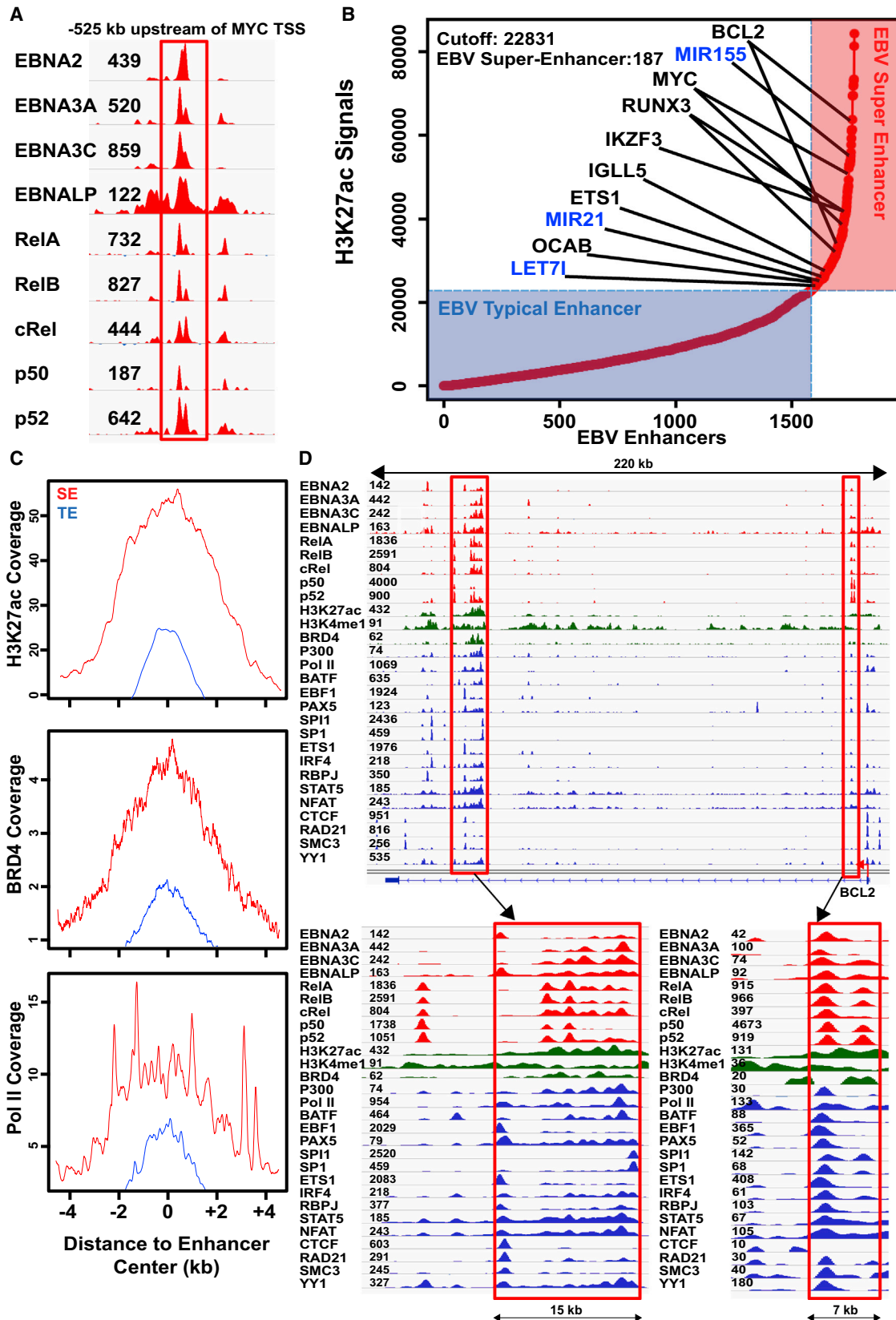


**Figure 1. EBNA2 Super-Enhancers**

(A) Rank order of EBNA2 ChIP-seq signals for all EBNA2 sites. Overall, 888 EBNA2 super-enhancer sites have >23-fold higher ChIP-seq signals than the average signals at ~17,000 other EBNA2 sites. These EBNA2 super-enhancer sites are annotated to their nearest cell gene. EBNA2 super-enhancer-associated cell genes particularly important for LCL growth or survival are indicated.

(B) EBNA2, other TFs, and histone modification ChIP-seq signals at super-enhancers near the *MYC* locus are shown. Numbers near gene names indicate tag density. A red arrow indicates the *MYC* TSS. Magnified views of EBNA2 super-enhancers (-525 and -428 kb) of *MYC* are shown (black arrows and red boxes).

(C) Average ChIP-seq signals for H3K27ac and H3K4me1 in 80 kb windows around EBNA2 super and typical enhancers are shown. Red indicates EBNA2 super-enhancers, and blue indicates typical enhancers.



(legend on next page)

Since unusually high and broad H3K27ac signals are indicative of super-enhancers, EBV enhancers were ranked based on their H3K27ac signals. Overall, 187 EBV enhancers had >4-fold higher H3K27ac signals than the rest of the EBV enhancers. These were therefore designated as “EBV super-enhancers” (Figure 2B; Table S2). In contrast to typical enhancers, which had average H3K27ac signals flanking a TF binding site without central elevation, the H3K27ac signals at EBV super-enhancer sites were broad and further elevated at the center of TF binding sites (Figure 2C).

In addition to H3K27ac, EBV super-enhancers also had much higher signals than typical enhancers for histone modifications, chromatin remodeling proteins, and basal transcription machinery, including H3K4me1 (3-fold), BRD4 (4.4-fold), Pol II (4.2-fold), BCLAF (3.6-fold), CHD1 (3.9-fold), MTA3 (3.8-fold), PML (4-fold), TAF1 (3.1-fold), and WHIP (3.9-fold) (Figures 2C and S1), proteins indicative of high super-enhancer transcription activity.

### EBV Super-enhancer-Associated Genes Are Important for LCL Growth and Survival

EBV super-enhancers were assigned to their target genes by proximity. A total of 96% of EBV super-enhancers and their regulated gene pairs resided within the same GM12878 LCL Hi-C TAD (Selvaraj et al., 2013). EBV super-enhancer-associated genes included *IGLL5*, *MYC*, *RUNX3*, *IKZF3/AIOLOS*, *ETS1*, *OCAB*, and *BCL2* (Figures 2B and 2D). *IGLL5* encodes Ig $\lambda$ , which has the strongest super-enhancer in myeloma cells (Lovén et al., 2013). *OCAB* is also controlled by a super-enhancer in DLBCLs (Chapuy et al., 2013), whereas *IKZF3* is an IKAROS family B cell TF that regulates B-cell proliferation. NF- $\kappa$ B-induced *BCL2* blocks apoptosis (Henderson et al., 1991). EBV super-enhancers were also associated with three miRNAs that are highly expressed in LCLs, including oncomir *MIR155*, *MIR21*, and *LET7I* (Figure 2B) (Skalsky et al., 2012).

Pathway enrichment analyses for EBV super-enhancer-associated genes identified enrichment for apoptosis, DNA damage response, and MAPK signaling pathways (Table S3).

EBV typical enhancer-associated genes included *TCF3/E2A*, *EBF*, *REL*, *IKZF1/IKAROS*, *BATF*, and *IRF4*, TFs important for B cell-specific transcription and B cell identity.

### EBV Super-Enhancer Enriched Motifs and Co-occurring Cell TFs

Super-enhancers are frequently enriched for cell type-specific TF motifs (Whyte et al., 2013). Similarly, EBV super-enhancers were significantly enriched for B cell-specific TF motifs, compared to the other control enhancers which were co-occupied by at least one EBV TF or NF- $\kappa$ B subunit but less than all nine. Enriched motifs included *MYC*, *SPI1*, *ETS1*, *STAT5*, *IRF4*, *RUNX*, *NFAT*,

*EBF*, *E2A*, and *SPI1/IRF4* composite site ( $p < 10^{-45}$ ) (Figure 3A). These motifs were also enriched when compared with EBV typical enhancers ( $p < 10^{-5}$ ). Even though many cell type-specific TF motifs are enriched in super-enhancers, only a smaller number of TFs distinguish super-enhancers from typical enhancers (Whyte et al., 2013). For example, *E2A* signals distinguish B cell super-enhancers from typical enhancers (Whyte et al., 2013). In LCLs, *NFAT* and *STAT5* signals at EBV super-enhancers were 5.1- and 3.8-fold higher than at typical enhancers ( $p < 2 \times 10^{-16}$ ) (Figure 3B). *NFAT* is implicated in B cell lymphomas (Pham et al., 2010), whereas *STAT5* is constitutively active in LCLs (Weber-Nordt et al., 1996). *STAT5* is also important for maintaining IL7 levels, which are critical for B cell development and survival (Clark et al., 2014). Enrichment for these TFs in EBV super-enhancers likely contributes to super-enhancer formation and function. Notably, *YY1* signals at EBV super-enhancers were 2.8-fold higher than at typical enhancers ( $p < 1 \times 10^{-16}$ ) (Figure 3B). *YY1* is important for long-range chromatin looping and transcription (Atchison, 2014). *YY1* motifs were only moderately enriched at EBV super-enhancer sites ( $p < 10^{-5}$ ), and only 11% of EBV super-enhancers had *YY1* motifs. Therefore, increased *YY1* binding at EBV super-enhancers was likely through interaction with other DNA binding proteins. EBV super-enhancers were also highly co-occupied by important B cell TFs, including *EBF* (100%), *BATF* (100%), *SPI1* (92%), *PAX5* (99%), *ETS1* (100%), and *IRF4* (100%) (Figures 3B and 3C).

### Comparison of EBNA and NF- $\kappa$ B Signals at EBV Super-Enhancers and Typical Enhancers

ChIP-seq signals for EBNA2, EBNA1P, EBNA3A, EBNA3C, and NF- $\kappa$ B subunits at EBV super-enhancers were compared to typical enhancers. EBNA2 signals at super-enhancers were 3.6-fold higher than at typical enhancers ( $p < 7.3 \times 10^{-12}$ ) (Figure 3D), whereas ChIP-seq signals for other EBV TFs or NF- $\kappa$ B subunits were far less significantly different or even similar between EBV super-enhancers and typical enhancers.

As expected, RBPJ motifs were also enriched ( $p < 10^{-28}$ ) at EBV super-enhancer sites, and RBPJ signals at EBV super-enhancer sites were 3.6-fold higher than at typical enhancers ( $p < 1.4 \times 10^{-10}$ ) (Figure 3D). EBNA2 can increase RBPJ DNA binding (Portal et al., 2011).

### EBV Super-Enhancer-Associated Genes Are Expressed at Significantly Higher Levels Than Typical Enhancer-Associated Genes

Expression levels of super-enhancer-associated genes are higher than typical enhancer-associated genes (Whyte et al., 2013). We therefore compared expression levels of EBV super-enhancer-associated genes with typical enhancer-associated genes

#### Figure 2. EBV Super-Enhancers

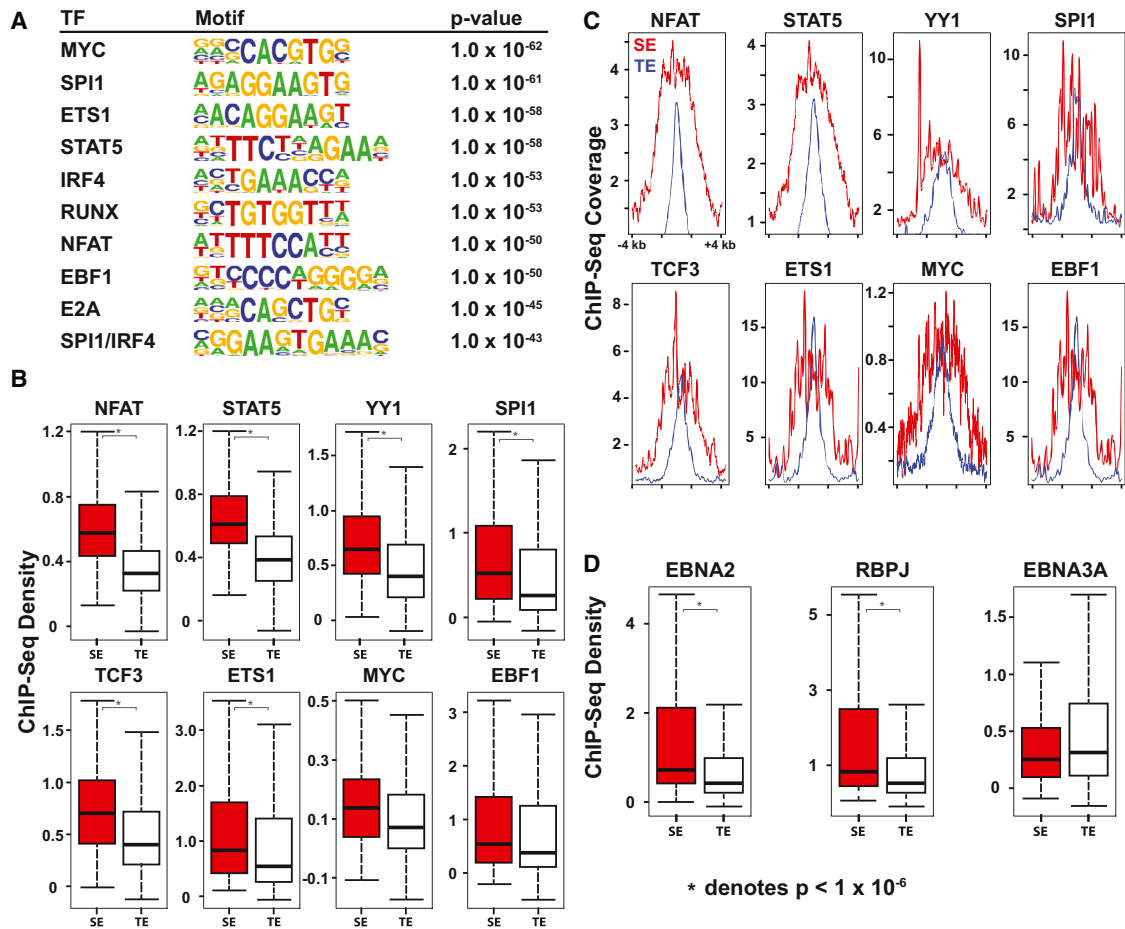
EBV super-enhancers are defined by high H3K27ac signals and the presence of all EBNA2 and NF- $\kappa$ B subunits.

(A) EBNA2, EBNA1P, EBNA3A, EBNA3C, NF- $\kappa$ B subunits RelA, RelB, cRel, p50, and p52 are all significantly present at the EBNA2 super-enhancer –525 kb of *MYC*.

(B) A total of 1,771 sites with significant EBV oncoproteins and NF- $\kappa$ B subunit binding are ranked by H3K27ac signals. A total of 187 EBV super-enhancers with 4-fold higher H3K27ac signals than EBV typical enhancers are annotated to their nearest genes. Genes important for LCL growth and survival are indicated.

(C) Anchor plots for H3K27ac, BRD4, and Pol II show substantially higher and broader signals (normalized coverage) at EBV super-enhancers than at EBV typical enhancers.

(D) ChIP-seq signals for virus and cell TFs and histone modifications at the *BCL2* locus.



**Figure 3. Transcription Factors that Differentiate EBV Super-Enhancers from EBV Typical Enhancers**

(A) TF motifs enriched at EBV super-enhancers over other control enhancers. Other control enhancers have more than one EBV TF or NF- $\kappa$ B subunit but fewer than all nine.

(B) Boxplots of TF ChIP-seq signals density at EBV super-enhancers and typical enhancers. Signals for NFAT, STAT5, YY1, and ETS1 at EBV super-enhancers are significantly higher than typical enhancers (Wilcoxon rank-sum test p values for the following: NFAT,  $p < 2 \times 10^{-16}$ ; STAT5,  $p < 2 \times 10^{-16}$ ; YY1,  $p < 1 \times 10^{-14}$ ; and ETS1,  $p < 3.8 \times 10^{-6}$ ; asterisk indicates  $p < 10^{-6}$ ).

(C) Anchor plots of normalized TF ChIP-seq signals (coverage) around EBV super-enhancers and EBV typical enhancers.

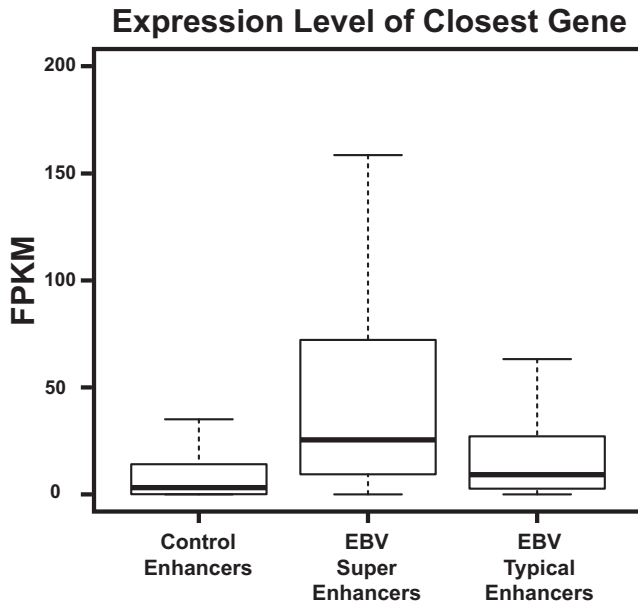
(D) Boxplots of EBNA2, RBPJ, and EBNA3A ChIP-seq signals (density) at EBV super-enhancers and typical enhancers. EBNA2 and RBPJ ChIP-seq signals at EBV super-enhancers are significantly higher than EBV typical enhancers (Wilcoxon rank-sum test p values are as follows: EBNA2,  $p < 7.3 \times 10^{-12}$ ; RBPJ,  $p < 1.4 \times 10^{-10}$ ; EBNA3A,  $p < 0.019$ ). For boxplots, middle line indicates the median. The edges indicate the first and the third quartile. The whiskers indicate minimum and maximum.

using RNA-seq data from three different LCLs that have minimal EBV replication (Arvey et al., 2012; Montgomery et al., 2010). Super-enhancer-associated genes were expressed at significantly higher levels than typical enhancer-associated genes (Wilcoxon rank-sum test,  $p < 3 \times 10^{-10}$ ) (Figure 4). Furthermore, EBV super-enhancer-associated gene expression levels and typical enhancer-associated gene expression levels were significantly higher than other control enhancer-associated gene expression levels (Wilcoxon rank-sum test,  $p < 2 \times 10^{-16}$ ).

### H3K27ac Signals at EBV Super-Enhancer Sites in LCLs and RBLs Have Similar Patterns

Previous analyses of LCL and RBL H3K4me1 signals at EBNA2 sites indicated that EBNA2 sites in LCLs and RBLs have remark-

ably similar patterns, although LCLs in general have higher H3K4me1 signals than RBLs (Zhao et al., 2011b). We therefore analyzed tonsil RBL H3K27ac signals (Chapuy et al., 2013) at EBV super-enhancer sites. Interestingly, in RBLs, these sites also had elevated H3K27ac signals, as compared with neighboring genomic regions. However, RBL H3K27ac signals were  $\sim 50\%$  lower than LCL signals (Figure 5A). The elevated RBL H3K27ac signals are indicative of pioneering B cell TF occupancy at these sites. Recruitment of p300, CBP, and PCAF histone acetyltransferases (HAT) or other chromatin remodeling proteins by EBV and EBV activated TFs such as EBNA2, EBNA3C, and NF- $\kappa$ B subunits may contribute to the increased H3K27ac signals (Perkins et al., 1997; Subramanian et al., 2002; Wang et al., 2000; Wu et al., 1996). Other control enhancers with less than all nine EBV TFs



**Figure 4. EBV Super-Enhancer-Associated Genes Have Higher Expression Levels Than Typical Enhancer-Associated Genes**

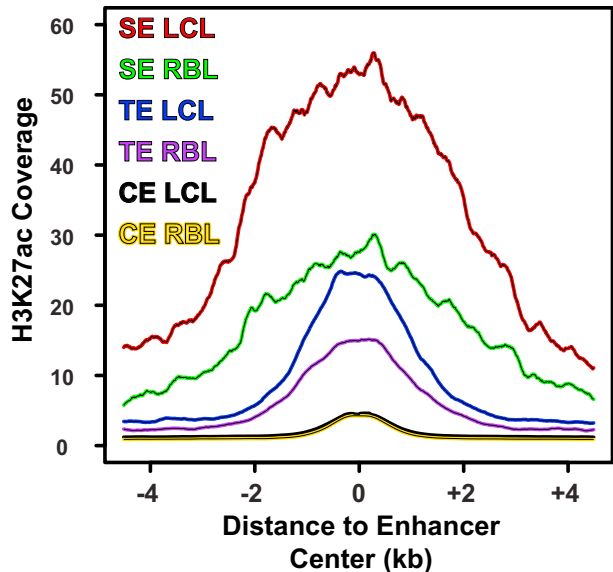
Boxplots of LCL EBV super-enhancer-associated, EBV typical enhancer-associated, and other control enhancer-associated RNA-seq gene expression levels (fragments per kilobase of exon per million fragments mapped [FPKM]) are shown. EBV super-enhancer-associated genes are expressed significantly higher than EBV typical enhancer-associated genes (Wilcoxon rank-sum test  $p < 3 \times 10^{-10}$ ). EBV super-enhancer-associated genes or EBV typical enhancer-associated genes are expressed significantly higher than other control enhancer-associated genes (Wilcoxon rank-sum test  $p < 2 \times 10^{-16}$ ). Middle line indicates the median. The edges indicate the first and the third quartile. The whiskers indicate minimum and maximum.

had much lower H3K27ac signals in both LCLs and RBLs (Figure 5A).

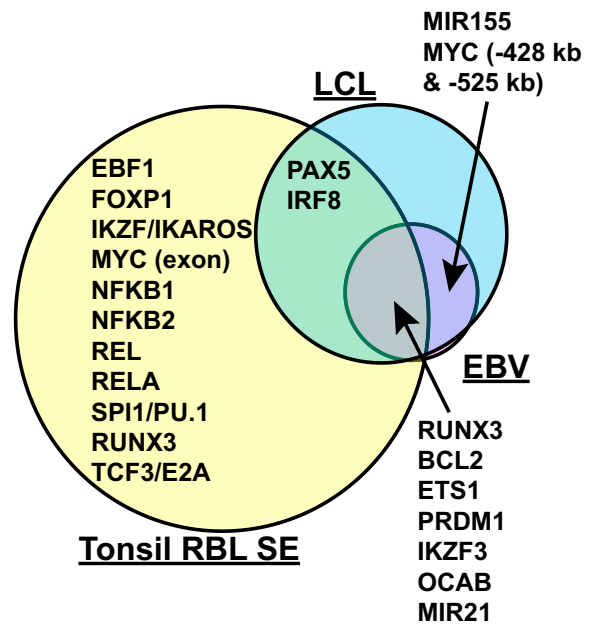
#### LCL and RBL Super-Enhancers

To compare LCL super-enhancers with RBL super-enhancers and identify super-enhancers in LCLs that are not co-occupied by all EBV TFs, tonsil RBL H3K27ac (Chapuy et al., 2013) and ENCODE GM12878 LCL H3K27ac ChIP-seq data were analyzed. The algorithm used to identify EBV super-enhancers identified 1,756 RBL and 655 LCL super-enhancers (Figures S2A and S2B). RBL super-enhancers were associated with most B cell-specific TFs or TFs important for B cell functions. These included *EBF*, *IKZF1/Ikaros*, *TCF3/E2A*, *PAX5*, *PRDM1/BLIMP1*, *IKZF3*, *OCAB*, *SPI1/PU.1*, *IRF8/ICSBP*, *RUNX1/3*, and *FoxP1*. *MYC* had a super-enhancer at a *MYC* exon in RBLs (Figures 5B, S2A, and S2B). Of LCL super-enhancers, 375 (57.3%) also overlapped with RBL super-enhancers. These included *PAX5*, *IRF8*, *RUNX3*, *PRDM1*, *IKZF3*, and *OCAB*. LCLs lost the *MYC* exon super-enhancer. Instead, LCLs gained two super-enhancers >400 kb upstream of *MYC*. LCLs also gained a super-enhancer at *MIR155*. The vast majority (~97%) of EBV super-enhancers were also LCL super-enhancers (Figures S3A–S3C). However, in LCLs, even though *PAX5* and *IRF8* were still linked to super-enhancers, they were not targeted by all EBV TFs. RBL and LCL super-

#### A Anchor Plots of Enhancers in LCLs vs RBLs



#### B



**Figure 5. H3K27ac Signals at EBV Super-Enhancer and Typical Enhancers in LCLs and RBLs**

(A) GM12878 LCL and tonsil RBL H3K27ac normalized coverage  $\pm$  4 kb of EBV super-enhancers (SE), typical enhancers (TE), and other control enhancers (CE) are shown.

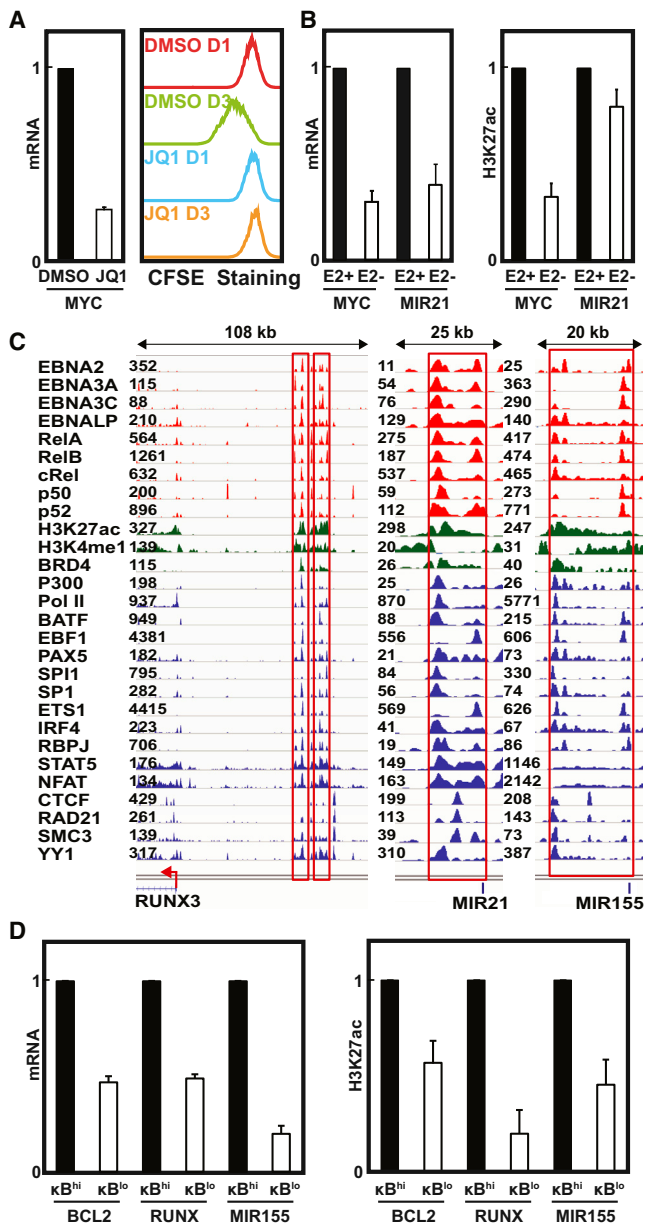
(B) Overlap between RBL, LCL, and EBV super-enhancer-associated genes. Genes important for B cell or LCL functions are indicated.

enhancer-associated genes were enriched for B cell functions (Table S4).

#### EBV Super-Enhancers Are Sensitive to Perturbation

BRD4 binds to acetylated histone lysine residues and further recruits CyclinT and CDK9 to phosphorylate Pol II and activate transcription. The BET bromodomain small molecule inhibitor





**Figure 6. Perturbation of Super-Enhancer Constituents Reduces Cell Growth, Gene Expression, and H3K27ac Signals**

All error bars represent SD.

(A) (Left) Twenty-four hours post-DMSO or 500 nM JQ1 treatment of GM12878 LCLs, MYC mRNA levels were measured using qRT-PCR and normalized to GAPDH. MYC mRNA levels in DMSO treated cells were set to 1. (Right) CFSE staining of GM12878 LCLs treated with DMSO or 500 nM JQ1 for 1 or 3 days. (B) (Left) Normalized MYC and MIR21 RNA levels, in conditional EBNA2 LCLs, grown under permissive (+) or nonpermissive (-) conditions for EBNA2 expression. (Right) H3K27ac levels at the -525 kb MYC and MIR21 EBV super-enhancers, as determined by ChIP qPCR. EBNA2 (+) condition was set to 1.

(C) EBV super-enhancers associate with *RUNX3*, *MIR21*, and *MIR155*. Super-enhancers are highlighted by red lines with TFs signals indicated on the left. (D) (Left) Normalized *BCL2*, *RUNX3*, and *MIR155* mRNA levels in LCLs with high NF- $\kappa$ B activity (hi) versus low NF- $\kappa$ B activity (lo). (Right) H3K27ac ChIP qPCR at *BCL2*, *RUNX3*, and *MIR155* EBV super-enhancers. The NF- $\kappa$ B hi condition was set to 1.

JQ1 specifically blocks BRD4 binding to acetylated lysine (Filippakopoulos et al., 2010) and hence disrupts DLBCL super-enhancer activity (Chapuy et al., 2013). Since BRD4 signals were evident at EBV super-enhancers and JQ1 disruption has been a super-enhancer hallmark (Figures 1B and 2D), the effect of JQ1 treatment on EBV super-enhancer activity was tested. GM12878 LCLs were treated with 500 nM JQ1 or DMSO vehicle for 24 hr. MYC expression was evaluated by quantitative RT-PCR. JQ1 treatment reduced MYC expression by more than 60% ( $p < 0.004$ ) (Figure 6A). JQ1 treatment also halted LCL growth, as indicated by carboxyfluorescein diacetate, succinimidyl ester (CFSE) staining (Figure 6A), indicating that EBV super-enhancers are similar to cell super-enhancers in sensitivity to BRD4 inhibition.

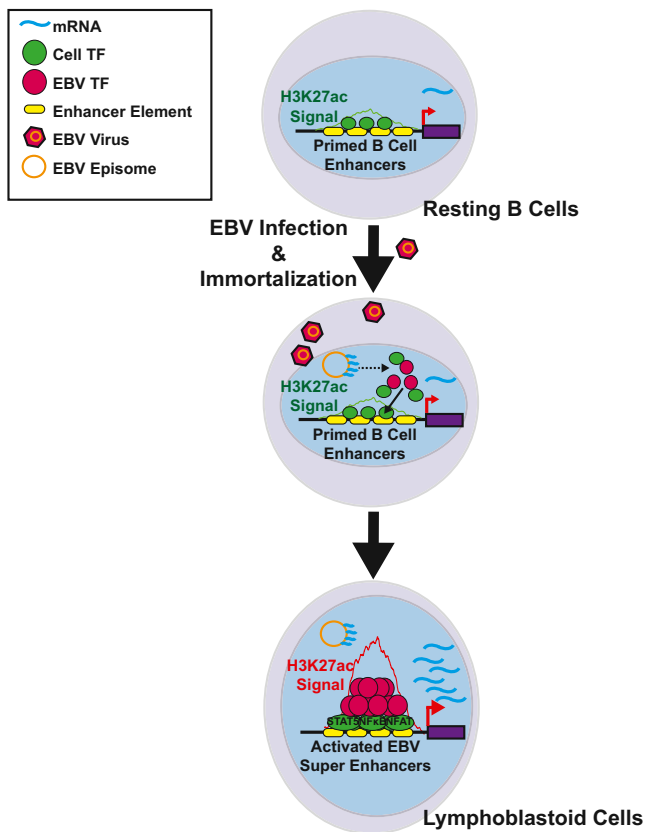
LCLs transformed by a recombinant EBV that express a conditional EBNA2, grow normally under permissive conditions for EBNA2 expression (Zhao et al., 2006). When these LCLs are grown under nonpermissive conditions for EBNA2, EBNA2 levels decrease and LCLs enter growth arrest (Zhao et al., 2006). Using these LCLs, we found *MYC* and *MIR21* RNA levels were reduced by ~60%–70% at 72 hr after EBNA2 inactivation ( $p < 0.0005$ ). Concurrently, ChIP-qPCR found *MYC* and *MIR21* super-enhancer H3K27ac signals were also significantly reduced ( $p < 0.05$ ) (Figure 6B).

Likewise, all NF- $\kappa$ B subunits were evident at *BCL2*, *RUNX3*, and *MIR155* super-enhancers (Figures 2D and 6C). Induction of a conditional  $\kappa$ B $\alpha$  dominant-negative mutant in LCLs inactivates NF- $\kappa$ B (Cahir-McFarland et al., 2000). NF- $\kappa$ B inactivation resulted in an ~50%–80% reduction in *BCL2*, *RUNX3*, and *MIR155* gene expression ( $p < 0.0005$ ) and caused a ~40%–80% reduction in H3K27ac signals at *BCL2*, *RUNX3*, and *MIR155* EBV super-enhancer sites ( $p < 0.05$ ) (Figure 6D). Furthermore, basal promoter luciferase reporters under the control of four different EBV super-enhancers or three different EBV typical enhancers were transfected into these LCLs. Luciferase activities were determined in LCLs with wild-type or reduced NF- $\kappa$ B activity. Inactivation of NF- $\kappa$ B reduced super-enhancer reporter activity by more than 40% and typical enhancer reporter activity by less than 20% ( $p < 0.02$ ) (Figure S4), indicating that EBV super-enhancers were more sensitive to NF- $\kappa$ B inactivation than EBV typical enhancers.

## DISCUSSION

B cell-specific tissue imprinting and associated TFs maintain B cell precursors in the bone marrow. EBF expression and pioneering effects of other B cell lineage TFs, IKZF1, SPI1, and PAX5, coordinately establish mature B cell identity and control gene expression (Lin et al., 2010). Subsequent antigen binding to B cell receptors and T cell CD40 ligand stimulation mediate RBL transcription activation.

During primary EBV infection, EBV transforms B cells into proliferating blasts, some of which ultimately differentiate into memory B cells. Latent EBV infection of the B cell compartment enables lifelong EBV infection. EBV-infected B cells can cause lymphomas in immune-suppressed individuals. This phenomenon is recapitulated in vitro, where EBV oncoprotein expression converts RBLs into LCLs. Our data indicate that EBV evolved to usurp B cell-intrinsic programs



**Figure 7. EBV Super-Enhancer Model**

RBLs have broad enhancer regions with moderate H3K27ac signals. These enhancers are occupied by a limited repertoire of cell TFs to maintain chromatin accessibility. Upon EBV infection, EBNA and LMP1 oncoproteins are expressed. LMP1 activates NF- $\kappa$ B. All EBNAs and NF- $\kappa$ B subunits then co-occupy primed B cell enhancer sites, recruit additional cell TFs, and nucleate EBV super-enhancers to upregulate transcription.

to support rapid growth and survival of latently infected B cells (Figure 7).

We herein identify a class of EBV super-enhancers that are comprised of EBV-encoded, EBV-activated, and EBV-associated B cell TFs. These EBV super-enhancers have exceptionally high signals for activation-associated histone modifications, Pol II, and chromatin remodeling factors, indicative of highly active transcription. EBV super-enhancers were associated with EBF, a principal pioneering B cell lineage factor, which increases chromatin accessibility, STAT5 and NFAT, which may nucleate EBV super-enhancer formation. These EBV super-enhancers likely exploit their high YY1 binding to loop to the TSS of affected genes, including genes critical for B cell growth and survival. EBV super-enhancers were sensitive to BRD4 perturbations as well as to EBNA2 and NF- $\kappa$ B inactivation.

EBV efficiently transforms RBLs within a week after infection. Thereafter, infected RBLs replicate every 24 hr, in vitro (Nikitin et al., 2010). Similarly, EBV-infected cells can replicate continuously in immune-deficient humans and express the same EBV genes as LCLs (Young et al., 1989). The convergence of all the EBV oncoproteins and EBV activated NF- $\kappa$ B subunits at

EBV super-enhancers has now been dynamically demonstrated to be a key determinant of continuous LCL growth.

EBNA2 is the principal EBV super-enhancer component that upregulates *MYC*. *MYC* overexpression is frequently the result of distal strong enhancers that loop to the *MYC* TSS. In prostate, breast, and colon cancers, the 8q24 cancer risk variant rs6983267, which is  $-335$  kb from *MYC* TSS, preferentially binds TCF7L2 and loops to *MYC* (Pomerantz et al., 2009). Similarly, EBNA2 mediates looping from a  $-428$  kb *MYC* enhancer to *MYC* (Zhao et al., 2011b). Another EBV super-enhancer which is  $\sim 525$  kb upstream of *MYC* is also likely to affect *MYC* activation. A long noncoding RNA 515 kb upstream of *MYC* can also loop to the *MYC* rs6983267 enhancer and affect enhancer activity (Xiang et al., 2014). Therefore, *MYC* expression is complex, and likely involves multiple distinct super-enhancers.

*MYC* overexpression-induced cell cycle entry causes apoptosis in the absence of strong prosurvival effects from BCL2 or activated tyrosine kinase growth factor receptors. EBV-associated Burkitt's lymphomas overexpress *MYC* as a consequence of chromosome translocations placing *MYC* under control of strong immunoglobulin enhancers. To overcome *MYC*-induced apoptosis, p53 is mutated in  $>50\%$  of Burkitt's lymphomas, and B cell lymphomas in *MYC* transgenic mice also often have inactivated p53 or ARF (Eischen et al., 1999; Love et al., 2012). In LCLs, two EBV super-enhancers upregulate BCL2 expression and thereby prevent *MYC*-induced apoptosis in LCLs. Lymphomas with both *MYC* and BCL2 overexpression have very poor clinical outcomes (Hu et al., 2013).

EBV infection of primary B cells upregulates *MIR155*, *MIR21*, and *LET7I*, which are important for LCL growth (Skalsky et al., 2012). *MIR155* is essential for LCL proliferation. EBV super-enhancers, enriched with EBNA2, EBNA1P, and LMP1-activated NF- $\kappa$ B subunits, upregulate *MIR155* and *MIR21* expression in LCLs (Rosato et al., 2012; Yang et al., 2013). *MIR21* regulates PTEN, and *LET7I* is associated with high-grade lymphomas (Lawrie et al., 2009). Control of these critical mirRNAs by EBV super-enhancer likely ensures LCL growth and survival.

Chromosome conformation capture carbon copy assays suggest that as few as 7% of enhancers regulate their nearest genes, although this can vary substantially among cell types (Sanyal et al., 2012). In contrast,  $>90\%$  of super-enhancers affect their nearest gene by Hi-C (Whyte et al., 2013). In support of most EBV super-enhancers also regulating the nearest promoter, we found that perturbation of EBV super-enhancers very frequently reduced expression of the nearest LCL genes and associated super-enhancer H3K27ac signals. These findings further correlated EBV super-enhancers to their nearby regulated genes.

Super-enhancers are highly accessible open chromatin regions with multiple co-occurring cell TFs. Although "accessible" loci may be prone to artifacts (Teytelman et al., 2013), EBV super-enhancers are specifically composed of EBNA2, EBNA1P, EBNA3A, EBNA3C, EBV-activated NF- $\kappa$ B subunits, and other EBV protein-associated cell TFs, as well as cofactors, active chromatin regulators, BRD4 and H2AZ, and core TFs. It is highly unlikely that EBV super-enhancers are due to ChIP-seq "artifacts," since dynamic perturbation of these super-enhancers by conditional EBNA2 or NF- $\kappa$ B inactivation led to

downregulation of EBV super-enhancer-associated genes and cessation of cell growth. Furthermore, EBV super-enhancers are functionally relevant to LCL biology.

EBNA2 inactivation substantially decreased MYC expression, but had surprisingly little effect on BCL2 expression, whereas NF- $\kappa$ B inactivation had a dramatic effect on BCL2 expression and small effect on MYC expression, confirming that MYC and BCL2 are differentially affected by EBNA2 and NF- $\kappa$ B, respectively. Increased activities from other EBV super-enhancer constituents are likely to compensate for the deficiency. Thus, the co-occurrence of other EBV TFs and NF- $\kappa$ B subunits at MYC and BCL2 may be indicative of an underlying, fail-safe transcription mechanism that assures proliferation and survival.

Like other super-enhancers (Lovén et al., 2013), EBV super-enhancers were sensitive to perturbations. JQ1 inhibition decreased super-enhancer-associated transcription more than typical enhancer-associated transcription (Chapuy et al., 2013; Lovén et al., 2013). As reflected in BRD4 inhibition and EBNA2 and NF- $\kappa$ B inactivation, EBV super-enhancers were sensitive to perturbation. These data indicate that EBV super-enhancer disruption may be effective in controlling EBV-transformed cell growth.

## EXPERIMENTAL PROCEDURES

### Sequencing Data Alignment

All ChIP-Seq reads were mapped to hg19 using Bowtie (version 0.12.9). Alignments were done with parameters: -S -t -p 1 -k 1 -m 1.

### Identifying ChIP-Seq-Enriched Regions

MACS (1.4.2) was used to identify ChIP-seq TF binding sites. Default parameters were used with the exception of “to-large,” which was set due to low sequencing depth of older ChIP-seq data sets.

### Motif Enrichment

HOMER (4.4) “findMotifsGenome.pl” was used to identify enriched motifs (Heinz et al., 2010) with “other control enhancers” set as background.

### Identification of Overlapping Binding Sites

HOMER “MergePeak” was used to identify the co-occurrence of binding sites (default parameters). If there was an overlap between the start and end coordinates of both enhancer regions, these enhancers were considered “overlapping.”

### Definition of EBV Enhancers

EBV enhancers were defined by the colocalization of four EBNA2s and five NF- $\kappa$ B subunits. Binding sites of these transcription factors were identified by MACS. “Other control enhancers” were defined by the presence of at least one, but less than all nine, TFs.

### Identifying EBV Super-Enhancers and EBNA2 Super-Enhancers

To identify EBV super-enhancers, all EBV enhancers were ranked according to their total background-subtracted H3K27ac ChIP-seq signal. EBV enhancers were sorted and plotted based on H3K27ac signals in ascending order. The x axis shows H3K27ac ChIP-seq signals’ rank order; the y axis shows normalized H3K27ac signals. A line was drawn from the first enhancer with lowest signal to the last enhancer with the highest signal to determine a diagonal slope. A point on the ranked plot with a tangent line identical to the diagonal slope was identified. This x axis point was set as the cutoff to distinguish EBV super-enhancers from EBV typical enhancers. The EBV enhancers with H3K27ac signals higher than this point were assigned as EBV super-enhancers.

EBNA2 super-enhancers were identified as described above using EBNA2 ChIP-seq signals. EBNA2 binding sites within 12.5 kb were stitched together as previously described (Whyte et al., 2013).

### Hi-C Validation of Super-Enhancer, and Associated Gene Assignment

GM12878 LCL Hi-C topological association domains (generated by Bing Ren Lab) (Selvaraj et al., 2013) were used to validate the assignment of super-enhancer and associated gene pairs. Correct assignments were scored based on the co-occurrence of a super-enhancer and its associated gene within the same TAD.

### Anchor Plots

Anchor plots show the distribution of TFs and histone modification ChIP-Seq signals at various binding sites, as previously described (Portal et al., 2013).

### Gene Expression Analysis

EBV super-enhancer-, EBV typical enhancer-, and EBV other control enhancer-associated gene expression was determined using LCL RNA-seq data. The average gene expression level (FPKM) was calculated with three LCLs (NA06985, NA07000, and NA07347). RNA-seq data from these three LCLs were chosen because they had the least amount of EBV late gene expression. Boxplots were drawn using R, and the statistical significance of the difference between each two pairs of the three groups was determined using the Wilcoxon signed-rank test.

### Pathway Enrichment Analysis

The enriched pathways of super-enhancer-associated genes were identified using the “Identify Pathways” function of the IntPath database (Zhou et al., 2012) and DAVID database (Huang et al., 2008).

### Reporter Assays

Four EBV super-enhancer and three typical enhancer sequences were PCR amplified and cloned into pGL3 promoter luciferase reporter vectors (Promega). A total of 20  $\mu$ g of control or enhancer luciferase vectors, together with 2  $\mu$ g of Renilla expression vector, was electroporated (Gene Pulser II, Bio-Rad), into  $7 \times 10^6$  LCLs containing an inducible  $\kappa$ B $\alpha$  mutant. Electroporated cells were split into permissive or nonpermissive conditions for mutant  $\kappa$ B $\alpha$  expression and grown for 48 hr. Dual luciferase/Renilla assays were done following the manufacturer’s directions (Promega).

### ACCESSION NUMBERS

BRD4 ChIP-seq data have been deposited to the Gene Expression Omnibus under accession number GSE62912.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and four tables and can be found with this article at <http://dx.doi.org/10.1016/j.chom.2014.12.013>.

### AUTHOR CONTRIBUTIONS

B.Z. and E.K. designed the study; B.Z. and E.K. supervised research; B.Z., S.C.S.S., S.J., B.W., K.B., B.E.G., and J.L. performed experiments; E.C.J. and P.K., provided new reagents; H.Z. performed the computational analysis; H.Z. and S.J. prepared figures and tables; and B.Z., H.Z., S.C.S.S., S.J., B.E.G., and E.K. wrote the manuscript.

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