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VIRUS-CELL INTERACTIONS

Epstein-Barr Virus Facilitates Expression of KLF14 by Regulating the Cooperative Binding of the E2F-Rb-HDAC Complex in Latent Infection

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ABSTRACT Epstein-Barr virus (EBV) was discovered as the first human tumor virus more than 50 years ago. EBV infects more than 90% of the human population worldwide and is associated with numerous hematologic malignancies and epithelial malignancies. EBV establishes latent infection in B cells, which is the typical program seen in lymphomagenesis. Understanding EBV-mediated transcription regulatory networks is one of the current challenges that will uncover new insights into the mechanism of viral-mediated lymphomagenesis. Here, we describe the regulatory profiles of several cellular factors (E2F6, E2F1, Rb, HDAC1, and HDAC2) together with EBV latent nuclear antigens using next-generation sequencing (NGS) analysis. Our results show that the E2F-Rb-HDAC complex exhibits similar distributions in genomic regions of EBV-positive cells and is associated with oncogenic super-enhancers involving long-range regulatory regions. Furthermore, EBV latent antigens cooperatively hijack this complex to bind at KLFs gene loci and facilitate *KLF14* gene expression in lymphoblastoid cell lines (LCLs). These results demonstrate that EBV latent antigens can function as master regulators of this multisubunit repressor complex (E2F-Rb-HDAC) to reverse its suppressive activities and facilitate downstream gene expression that can contribute to viral-induced lymphomagenesis. These results provide novel insights into targets for the development of new therapeutic interventions for treating EBV-associated lymphomas.

IMPORTANCE Epstein-Barr virus (EBV), as the first human tumor virus, infects more than 90% of the human population worldwide and is associated with numerous human cancers. Exploring EBV-mediated transcription regulatory networks is critical to understand viral-associated lymphomagenesis. However, the detailed mechanism is not fully explored. Now we describe the regulatory profiles of the E2F-Rb-HDAC complex together with EBV latent antigens, and we found that EBV latent antigens cooperatively facilitate KLF14 expression by antagonizing this multisubunit repressor complex in EBV-positive cells. This provides potential therapeutic targets for the treatment of EBV-associated cancers.

KEYWORDS E2F-Rb-HDAC, Epstein-Barr virus, KLF14, latent infection

The E2F family is traditionally divided into several subgroups as activators (E2F1 to E2F3) or suppressors (E2F4 to E2F8) based on their transcriptional properties *in vivo* (1). E2Fs have also been described for their critical roles in regulating cell proliferation and cell cycle (2). These convergent studies revealed that E2Fs were the functional target of the first identified tumor repressor retinoblastoma (Rb) (3, 4). Importantly, the E2F/Rb pathway is also critical in regulation of cell growth and development of cancer (5, 6). Rb can block E2F transcription activation by binding to its transactivation domain

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(7, 8). Furthermore, Rb can interact with E2Fs and HDACs (histone deacetylase), simultaneously. Thus, the E2F/Rb complex suppresses transcription through the recruitment of HDACs, which contain an LXCXE motif required for their interaction with the domain B of the Rb protein (9–11). Recruitment of the E2F-Rb-HDAC complex can regulate chromatin structure by modifying histone acetylation and further inhibit transcription activity (11–13). Additionally, adenovirus E1A protein, simian virus 40 (SV40) large T antigen, and human papillomavirus-16 E7 oncoprotein bind the Rb pocket domain and inactivate its function to facilitate cell transformation (14–17). These results suggest that the cooperation of these cellular factors is important for the regulation of downstream signaling targets. For example, DNMT1, a predominant mammalian DNA methyltransferase, can bind the E2F-Rb-HDAC complex to repress the transcription activity of E2F-responsive promoters (18).

Epstein-Barr virus (EBV) is the first known human tumor virus and has been studied for more than 50 years (19–21). EBV infects more than 90% of the population worldwide and is associated with numerous diseases, including Burkitt's lymphoma (BL), Hodgkin lymphoma (HL), diffuse large B-cell lymphoma (DLBCL), nasopharyngeal carcinoma (NPC), and gastric carcinoma (GC) (22, 23). EBV is still the most efficient transforming virus capable of immortalizing human primary B lymphocytes *in vitro* (24). Two types of infection can be predominantly established in EBV-infected cells, latency in primary B lymphocytes and lytic infection in epithelial cells (22, 25). During latent infection, different latent programs are defined by the expression programs of the viral genome, which results in a specific repertoire of EBV proteins (24). This lifelong and persistent EBV infection in the infected host plays a critical role in driving EBV-associated tumorigenesis. EBV latent antigens can induce lymphomagenesis in B cells by dysregulating the transcription network and, thus, protein expression at multiple levels (24). Additional studies focus on the complicated transcription regulatory networks in EBV-induced B cell transformation *in vitro* (19). Super-enhancers are clusters of transcriptional enhancers bound by multiple transcription factors that are critical for cell identity or cell diseases (26). EBV super-enhancers with higher H3K27ac signals increase the expression of both *MYC* and *BCL2* genes to promote cell proliferation (27). EBV nuclear antigens (EBNAs) are essential for the organization of MYC super-enhancer-associated chromatin loops, which further increases MYC expression (28, 29). However, revealing the effects of chromosome modification on transcriptional regulatory networks, as well as EBV-induced B-cell transformation, does not provide the entire picture. How EBV oncogenes regulate the human three-dimensional (3D) genome and reprogram the cellular functions is still mostly an unexplored area.

As a classical signaling pathway, the E2F-Rb-HDAC complex can be controlled and utilized by EBV to facilitate its tumorigenic activities. For example, EBV immediate early protein BRLF1 also recruits the E2F family members to activate the EBV DNA polymerase promoter (30). Interestingly, another EBV immediate early protein, BZLF1, also induces E2F1 expression and other cell cycle-associated genes (31). LMP1 decreases p27 transcription by recruiting the repressive E2F4 protein to E2F sites within the p27 promoter (32). E2F1 transcription factor could also activate the EBV BamHI-F promoter (Fp) activity by antagonizing EBNA1-mediated transcription inhibition (33). Our previous studies showed that EBNA3C can interact with the Rb protein and mediates its degradation through an SCF (Skp1-Cul1-F-box-protein) ubiquitin ligase Skp2 (34, 35). EBNA3C mediates E2F1 downregulation and E2F6 upregulation to facilitate cell cycle progression (36, 37). Also, HDACs are strictly regulated and target multiple genes by modulating chromosome structure during EBV infection (38, 39). For instance, the myocyte enhancer-binding factor 2 (MEF2) recruits class II HDACs to the *BZLF1* gene promoter and changes the status of chromatin acetylation during latent infection (40). Furthermore, the histone deacetylase inhibitor romidepsin exerts strong antitumor activity via reducing LMP1 and c-Myc expression in EBV-positive DLBCL (41). Treatment with the HDAC inhibitor sodium butyrate regulates the switch from lytic to latent infection, which increases STAT3 expression (42). Therefore, HDAC inhibitors induce EBV lytic-phase gene expression and can be potently utilized for treatment of EBV-

associated lymphomas (43). However, whether EBV antigens target the E2F-Rb-HDAC complex to regulate organization of oncogenic super-enhancers is yet to be fully resolved.

Here, we investigated the binding characteristics of cellular factors E2F6, E2F1, Rb, HDAC1, and HDAC2 on the human genome to explore the role of EBV-mediated transcriptional regulatory networks in controlling the organization of super-enhancers. We found that EBV latent antigens cooperated with this repressor complex E2F-Rb-HDAC and promoted the expression of two new targets (KLF10 and KLF14) in EBV-positive cells. These results provide novel insights into the molecular mechanism and a deeper understanding of EBV-associated regulation which can be harnessed for development of novel treatment strategies.

RESULTS

The E2F, Rb, and HDAC exhibit similar distribution at annotated genomic regions in lymphoblastoid cell lines (LCLs). E2F-Rb-HDAC is a functional transcription regulatory module that modulates the expression of several specific target genes in human cancers (5, 44). Both E2F6 and E2F1/Rb can act as tumor repressors to inhibit gene expression through the recruitment of histone deacetylases 1 (HDAC1) or histone deacetylases 2 (HDAC2) (9–11, 45–47). Our previous studies demonstrated that EBV latent antigens interact with these molecules to regulate their expression and functional activities (34, 36–38). How these viral antigens modulate the downstream target genes through cooperative binding of the E2F-Rb-HDAC complex, and whether viral antigens can reverse their inhibition of expression of specific targets are not fully understood in EBV-infected cells.

To explore the detailed regulatory mechanisms of E2F-Rb-HDAC in the background of EBV, we first performed high-throughput chromatin immunoprecipitation sequencing (ChIP-Seq) analysis of the EBV-transformed lymphoblastoid cell line (LCL1) to identify the genomic regions enriched for these transcription regulatory factors, which included E2F6, E2F1, Rb, HDAC1, and HDAC2 (Fig. 1). The results from the ChIP-Seq analysis demonstrated that E2F6 and E2F1 as well as Rb primarily bound to noncoding DNA regions of the human genome, particularly introns and intergenic regions, while another 3% to 5% were regions which include transcription start sites and promoters associated with regulation of coding sequences (Fig. 1A). Similarly, HDAC 1 and 2 were enriched at introns and intergenic regions (Fig. 1A). E2F6 was shown to be enriched at approximately 3-fold the number of mutually exclusive binding sites compared to E2F1; however, the number of common binding sites shared by these factors in LCLs were much greater than the single sites (Fig. 1B). Additionally, HDAC1 and HDAC2 may indirectly bind the genomic sites through their association with E2Fs or Rb protein. Interestingly, HDAC2 is associated with approximately 90-fold more mutually exclusive binding sites than HDAC1 in LCL1 cells, although they have overlapping sites. These results suggest that HDAC2 has a prominent and multifunctional role in EBV-mediated lymphomagenesis (Fig. 1C).

To further investigate the binding characteristics, we determined the overlap of these five cellular factors that are located at enriched regions on the human genome. Our results showed that approximately 20,073 sites were bound similarly for E2F6, E2F1, Rb, HDAC1, and HDAC2 (Fig. 1D). Therefore, E2F-Rb-HDAC is a critical transcriptional functional complex, the parts of which are distributed similarly to each other owning many common binding sites in EBV-transformed LCL cells, which implies that they are tightly coordinated in the regulation of EBV latency.

The E2F-Rb-HDAC complex can function as a super-enhancer in LCLs. The E2F-Rb-HDAC signaling pathway that is linked to many functional activities can transcriptionally regulate its downstream targets (9, 10, 47). Following our determination of the patterns of their binding sites or regions in EBV-positive LCL cells, we found that E2F6, Rb, and HDAC2 could bind to very small regions of 100 to 200 bp, while E2F1 and HDAC1 bound to a relatively wider region which encompasses approximately 400 bp in size (Fig. 2, A and B). These protein complexes can only bind to a small region and have

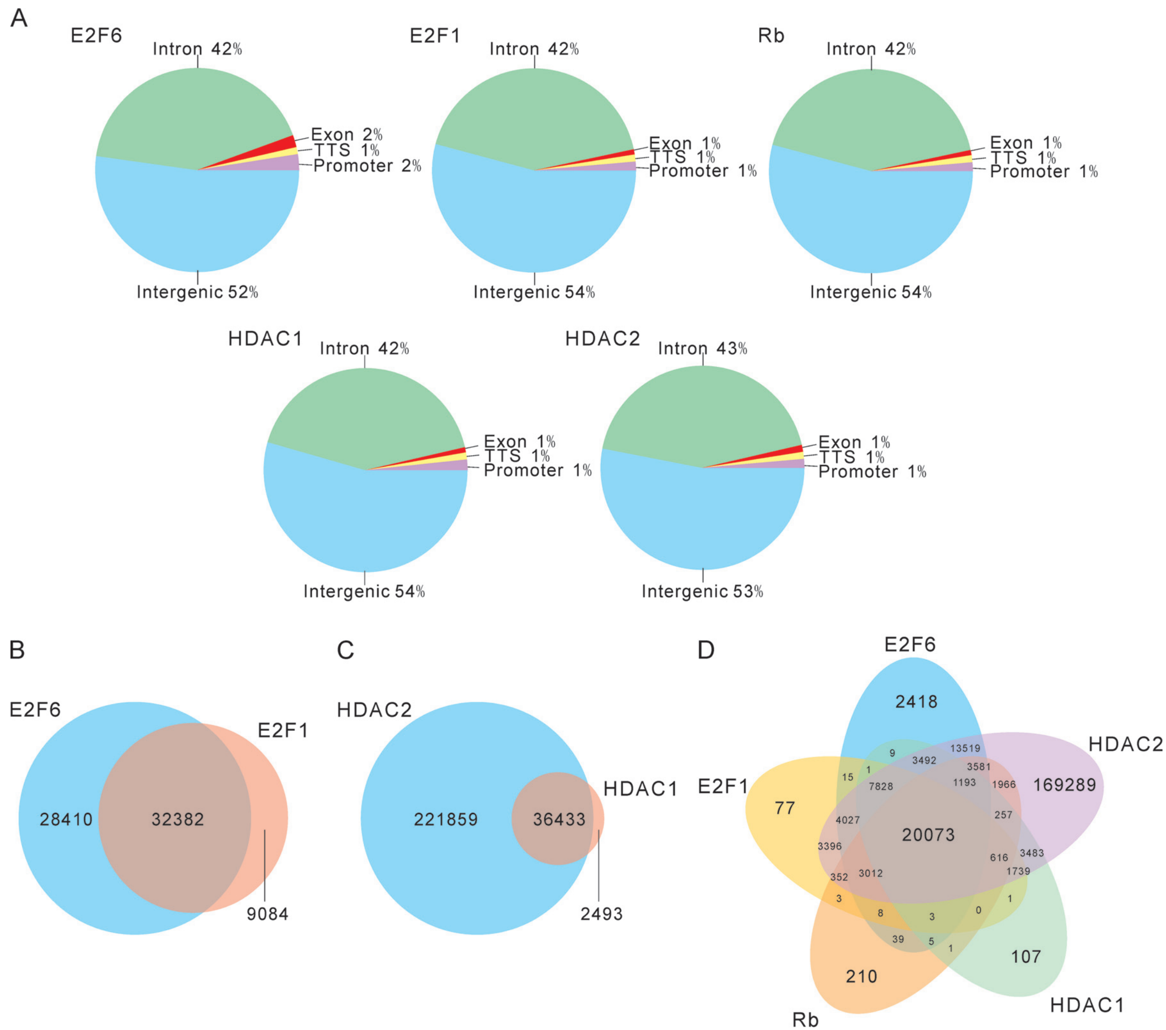

FIG 1 The E2F, Rb, and HDAC exhibit similar distribution at annotated genomic regions in LCLs. (A) Distribution of E2F6, E2F1, Rb, HDAC1, and HDAC2 occupancy at annotated genomic regions in EBV-transformed LCLs. TTS, transcription termination site; Exon, coding region. (B and C) Venn diagrams showing the overlap of E2F6 and E2F1 (B) and HDAC1 and HDAC2 (C) peaks in LCLs. The numbers indicate the binding sites of these cellular factors. (D) Overlap of E2F6, E2F1, Rb, HDAC1, and HDAC2 binding sites in LCLs. The numbers demonstrate the overlapping peaks of the indicated factors.

universal characteristics of binding to human genomic regions as a single binding factor. However, they may function in long-range regulatory regions when generating a multicomponent complex and coordinating with other proteins to perform a specific function in gene regulation. To detect whether E2F-Rb-HDAC can cooperate as a regulatory complex in LCL1 cells, super-enhancer analysis using the HOMER program was performed to identify their associated enhancers (48, 49). The results from our analyses identified super-enhancers (SE) as well as typical enhancers (TE) that were defined by the slope threshold of 1 (Fig. 2C). Furthermore, these five cellular transcription factors are more enriched at the super-enhancer sites than the typical enhancer sites in EBV-transformed LCLs (Fig. 2D), suggesting that the functions of these proteins are consistent with regulation of transcriptional networks from long-range enhancers, although they may need the assistance of additional recruiters.

The E2F-Rb-HDAC complex regulates shared downstream signaling pathways. To further investigate the cooperation of E2F-Rb-HDAC, we analyzed their regulated downstream signaling pathways with gene ontology analysis based on their binding regions. The results demonstrated that they are potentially involved in several common pathways (Fig. 3A to E). These cellular factors can target many common binding sites

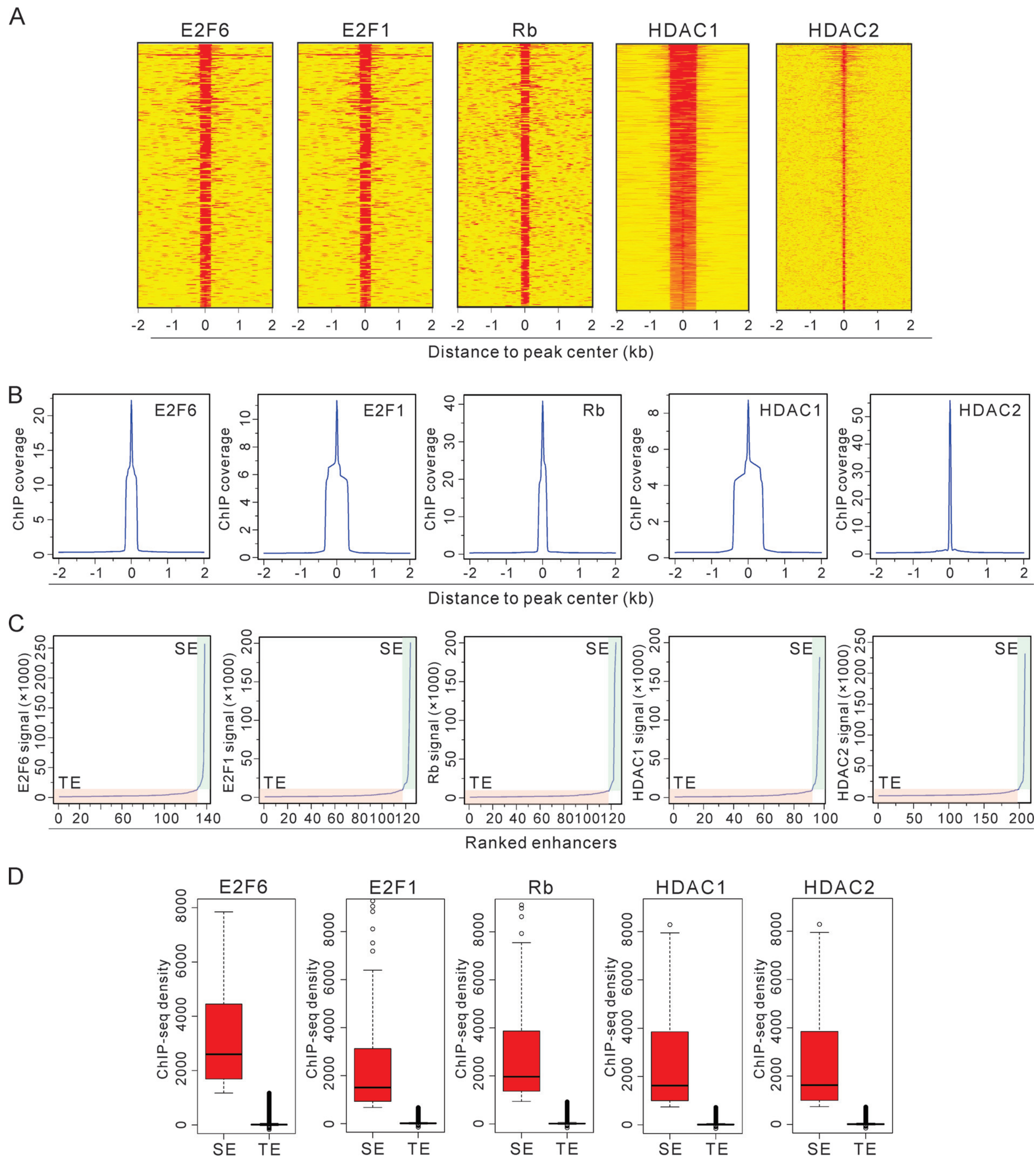

FIG 2 The E2F-Rb-HDAC complex can function as a super-enhancer in LCLs. (A and B) Heatmap view (A) and anchor plots (B) of E2F6, E2F1, Rb, HDAC1, and HDAC2 binding intensity at annotated human genome in LCLs. ChIP-Seq signals of the indicated cellular factors were created in a ±2-kb window. (C) The rank order of E2F6, E2F1, Rb, HDAC1, and HDAC2 ChIP-Seq signals for their enhancers with the module of "finding super enhancers" in the HOMER program. The typical enhancers (TE) and super enhancers (SE) are highlighted with light orange or blue, respectively. (D) Boxplots of E2F6, E2F1, Rb, HDAC1, and HDAC2 ChIP-Seq signal density at super-enhancers and typical enhancers. SE, super enhancers; TE, typical enhancers.

as shown in Results (Fig. 1D). More specifically, both E2F6 and E2F1/Rb are linked to the Wnt signaling network, regulation of RAC1 activity, the EphrinA-EPHA pathway, and BMP receptor signaling (Fig. 3F), which are tightly associated with EBV infection (50–54). Similarly, HDAC1, but not HDAC2, is also associated with the Wnt signaling network, suggesting that E2F-Rb interactions are more likely to recruit HDAC1 for the related regulation (Fig. 3D). Besides, the shared signaling pathway of HDAC1 and HDAC2 in

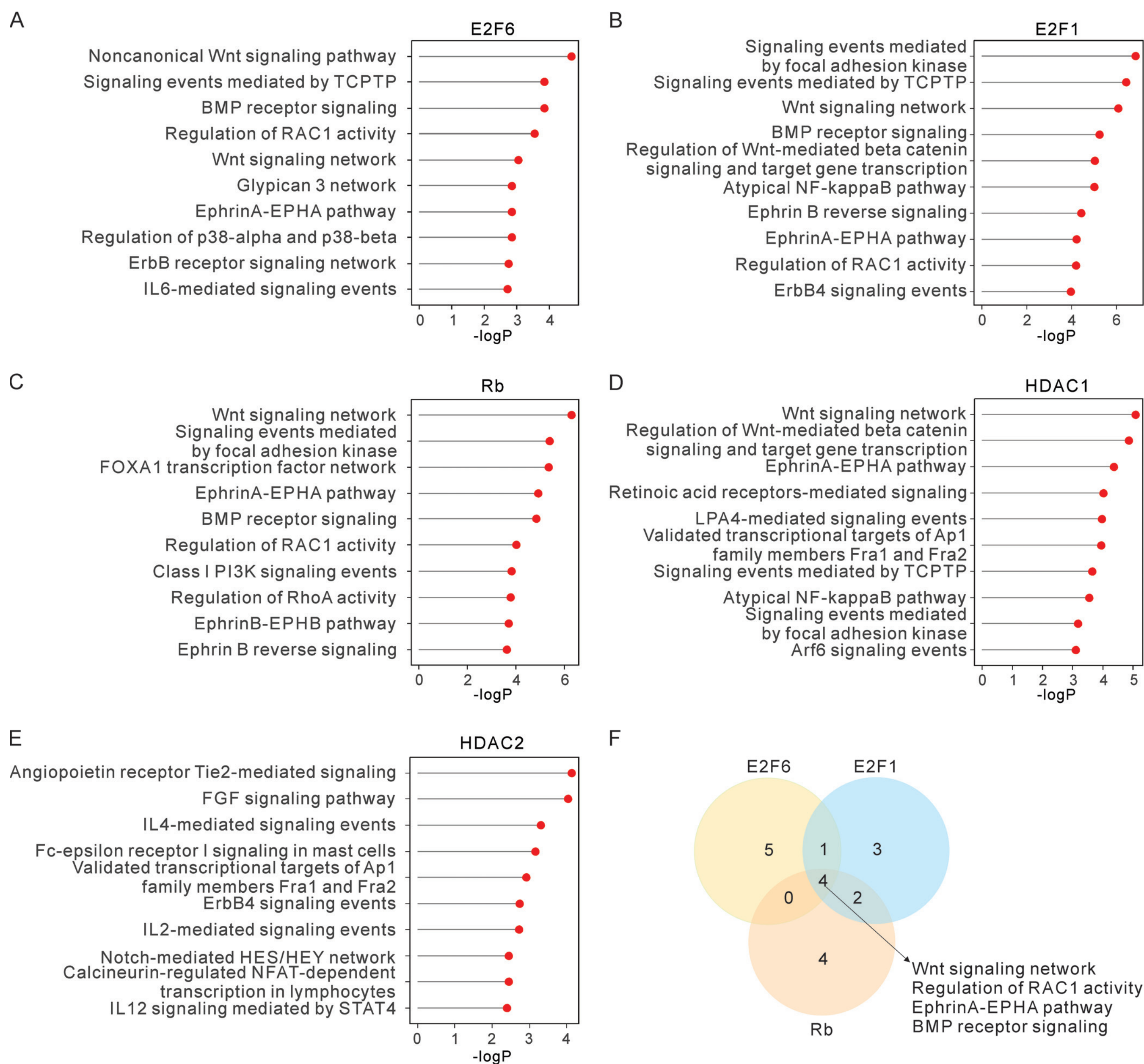

FIG 3 The E2F-Rb-HDAC complex regulates shared downstream signaling pathways. (A to E) Gene ontology analysis is conducted to annotate enriched peaks or regions with the ChIP-Seq data of E2F6 (A), E2F1 (B), Rb (C), HDAC1 (D), and HDAC2 (E) and shows the regulated signaling pathways by these cellular factors. GO annotation of the top 10 enriched signaling pathways was shown according to the indicated *P* value. (F) Venn diagram demonstrating the overlapped signaling pathways among E2F6, E2F1, and Rb in LCLs. Four shared signaling pathways of these three transcription factors (E2F6, E2F1, and Rb) were highlighted.

LCLs is related to AP1 family members Fra1 and Fra2 (Fig. 3D and E), which are also involved in EBV infection (55). Therefore, these results suggest that multiple common transcription regulatory patterns linked to these cellular factors are associated with EBV-induced B-cell lymphomas.

EBV regulates the binding of E2F6 and E2F1/Rb at KLF gene regions. E2F6 and E2F1 are the critical transcription factors in the E2F-Rb-HDAC complex that can directly bind to genomic DNA (2, 56, 57). To further explore the enriched genomic regions bound by these cellular factors, motif analysis was performed to identify their shared targets in EBV-positive LCLs. Among these binding motifs, we found that these five cellular transcription regulators could bind several common target genes, including

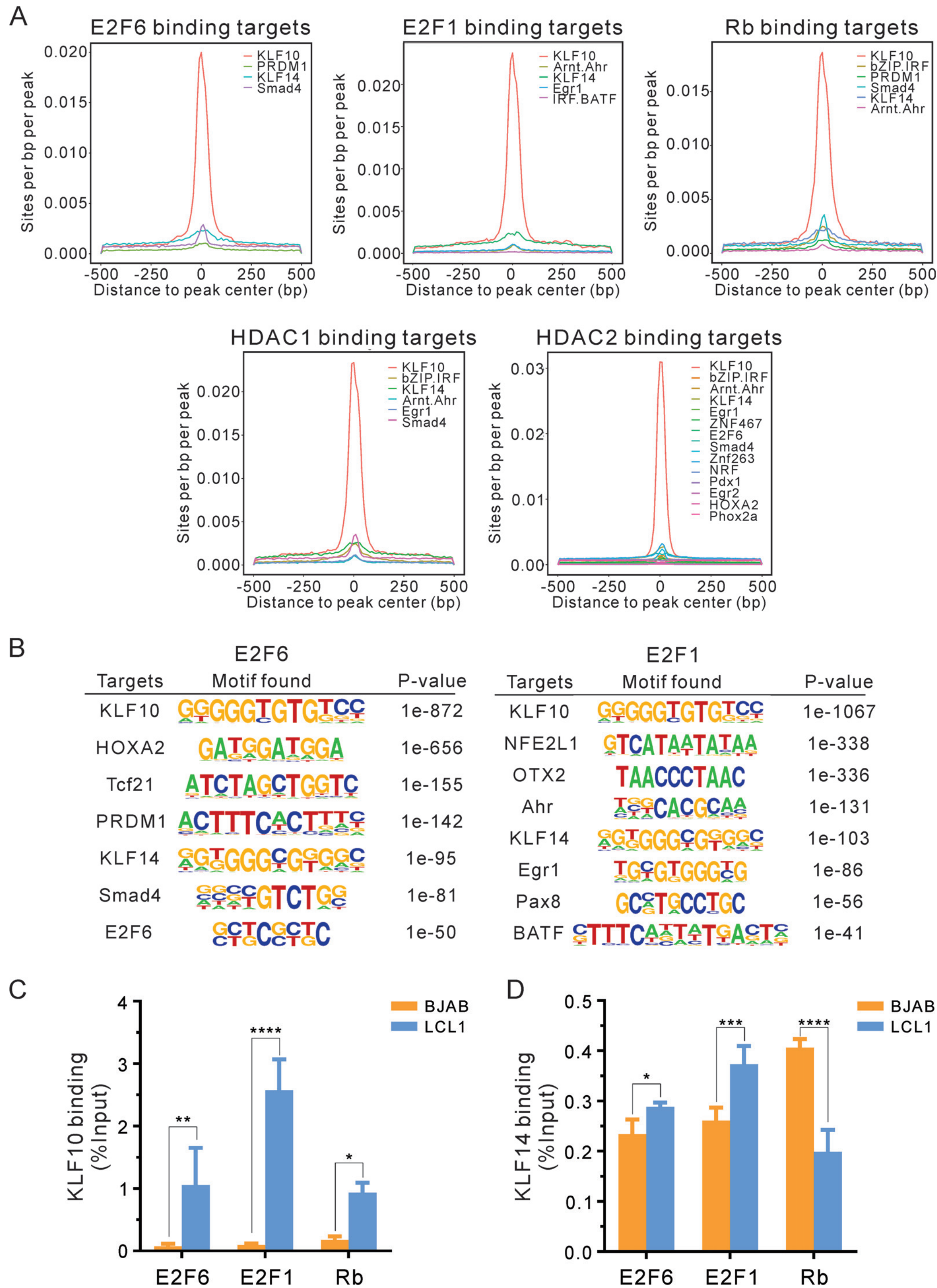

FIG 4 EBV regulates the binding of E2F6 and E2F1/Rb at KLF gene regions. (A) Anchor plots show the indicated cellular factor (E2F6, E2F1, Rb, HDAC1, and HDAC2) binding sites and targeted genes. ChIP-Seq signals of the cellular factors on the described targets were created in a ±500-bp window. (B) Motif analysis by HOMER presents the enriched binding motifs of E2F6 and E2F1 in LCLs. (C and D) ChIP experiments show the enrichment of E2F6, E2F1, and Rb at the coding region of KLF10 (C) or KLF14 (D) in BJAB and LCL1 cells. Results are the mean ± standard error of the triplicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

KLF10 and KLF14, which were two of the most significant targets as determined by *P value* (Fig. 4A and B). Therefore, these results demonstrated that the E2F-Rb-HDAC complex which contains E2F6 or E2F1/Rb, and HDAC1/HDAC2 showed several consistent patterns, such as binding sites, the length of enrichment regions, the associated downstream pathways, and multiple targeted genes.

KLF10 and KLF14 are two members of the Krüppel-like family of transcription factors (KLFs) (58). KLF10, also named TGF-β inducible early gene-1 (TIEG-1), is rapidly induced by TGF-β1 (59). Furthermore, TGF-β1 can be induced by EBV infection and this induction leads to activation of EBV lytic infection (60, 61). This suggests that KLF10 may be upregulated during EBV infection following the induction of TGF-β1. The functions of KLF14 and its potential roles in EBV-infected cells are not well-known. One study showed that KLF14 was necessary for the suppression of centrosome amplification and tumorigenesis (62). To verify the regulation of the E2F-Rb-HDAC complex on *KLF10* and *KLF14* genes, we performed chromatin immunoprecipitation (ChIP) assays to detect whether E2F6 or E2F1/Rb could bind to KLF10 and KLF14 loci in EBV-negative BJAB and EBV-positive LCL1 cells. Our results demonstrated that both E2F6 and E2F1/Rb could bind to both KLF10 and KLF14 coding regions. These three cellular factors (E2F6, E2F1, and Rb) were more enriched at the *KLF10* gene in LCL1 cells compared to that seen in BJAB cells (Fig. 4C). Additionally, E2F6 and E2F1 showed more enrichment at the *KLF14* gene in EBV-positive LCL1 cells than in EBV-negative BJAB cells, but Rb showed the opposite enrichment in these cell lines (Fig. 4D). The results suggested that the regulation of KLF10 and KLF14 may be mediated by distinct mechanisms in LCL1 cells. In particular, KLF10 expression is more dependent on Rb than KLF14 expression, which can be modulated by EBV latent antigens through directly targeting E2F6 or E2F1 without the assistance of Rb (36, 37). Therefore, these findings demonstrated not only that these proteins could bind to *KLF10* and *KLF14* genes in the B-cell background, but also that EBV latent antigens may coordinate with E2F6 or E2F/Rb to regulate the downstream genes which include *KLF10* and *KLF14*.

EBV latent antigens are responsible for KLF14 upregulation. In EBV latently infected B cells, the E2F-Rb-HDAC multifunctional repressor complex involved in crucial regulatory pathways controlled by EBV latent antigens (34, 36, 37, 63, 64). To determine whether KLFs are associated with EBV infection, we first explored the expression of KLF10 and KLF14 in EBV-transformed GM12878 cells from public data sets (65, 66). GM12878 is another EBV-transformed lymphoblastoid cell line that has been widely used in the International HapMap Project and the Encyclopedia of DNA Elements (ENCODE) Project (65, 66). By searching the histone modifications of the *KLF10* or *KLF14* gene in the ENCODE project, we found that the upstream regions of KLF10 or KLF14 coding sequences were highly enriched by H3K4me1, H3K4me2, and H3K4me3 modifications, which were related to activated promoters or enhancers (67) (Fig. 5A). Additionally, the RNA-Seq data indicated the peaks of *KLF10* or *KLF14* gene in EBV-transformed GM12878 cells (Fig. 5A). These results showed that *KLF10* and *KLF14* genes are associated with high expression in EBV-positive cells.

The expression and functional role of KLF14 in EBV-infected cells have not yet been elucidated. To determine KLF14 expression in the presence of EBV infection, quantitative real-time PCR analyses were conducted with different EBV-negative or EBV-positive B cell lines. The results showed that KLF14 expression was slightly downregulated in EBV-positive Raji cells compared to EBV-negative BJAB cells (Fig. 5B). The viral-encoded EBNA1 antigen is the most prominent latent antigen expressed in Raji cells, which has a deletion in the *EBNA3C* gene and lacks EBNA3C (68). This suggested that EBNA1 may function as a repressor of KLF14 mRNA expression in EBV latently infected B cells. Interestingly, two other EBV-positive cell lines (BL41/B95.8 and LCL1) expressed more KLF14 mRNA than the BJAB cell line (Fig. 5B). All EBV latent antigens were expressed in these two cell lines, therefore, this strongly suggests that EBV latent antigens, except for EBNA1 can significantly promote the upregulation of KLF14 mRNA expression in EBV-positive cells. To further validate our results, we detected KLF14 mRNA expression in other EBV latently infected cell lines, which were matched in latency I program (SavI, KemI, MutuI) only expressing EBNA1 antigen or in latency III program (SavIII, KemIII, MutuIII) expressing all EBV latent antigens. The results clearly showed that KLF14 mRNA was upregulated in EBV latency III cells compared to EBV latency I cells, which indicated

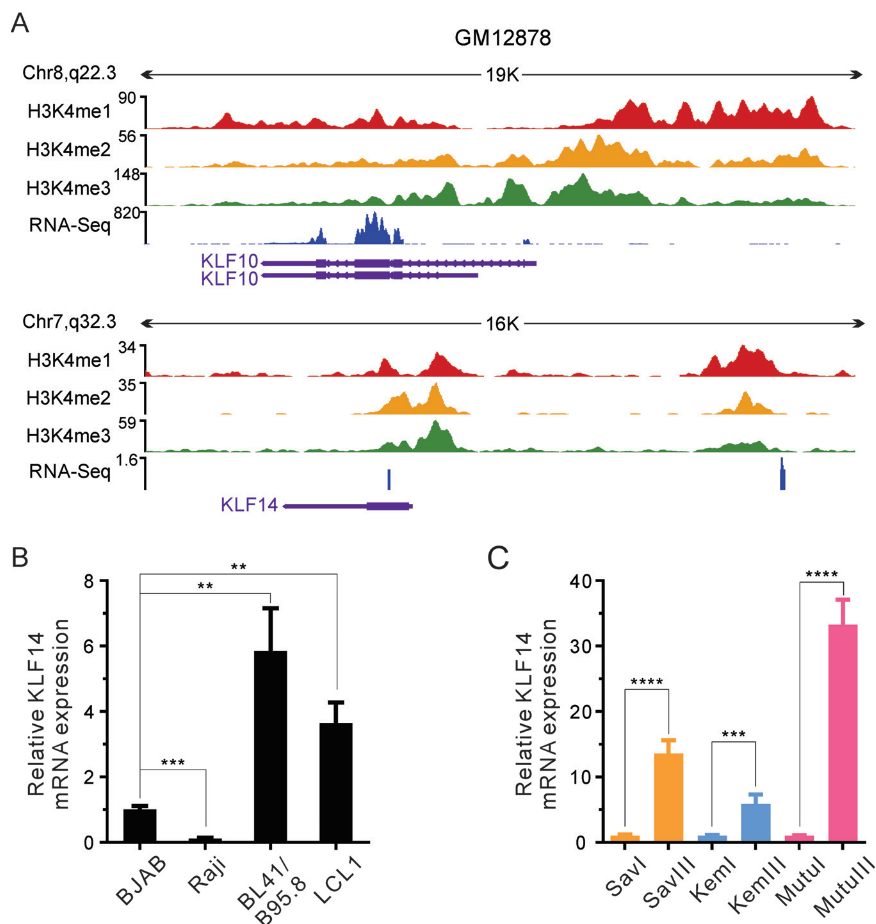

FIG 5 EBV latent antigens are responsible for KLF14 upregulation. (A) RNA-Seq from the ENCODE data set shows the mRNA expression of KLF10 or KlF14 in EBV-transformed GM12878 cells, while ChIP-Seq from the ENCODE data set shows the enrichment of histone modifications (H3K4me1, H3K4me, H3K4me3) at the *KLF10* or *KLF14* gene in GM12878 cells. (B) Real-time PCR analysis shows the level of KLF14 mRNA expression in BJAB (EBV-negative cell line), Raji (EBV-positive cell line expressing only EBNA1 antigen), BL41/B95.8 and LCL1 (EBV-positive cell lines expressing all of the EBV latent antigens) cells. Results are the mean ± standard error of the triplicates. **, $P < 0.01$; ***, $P < 0.001$. (C) Real-time PCR analysis shows KLF14 mRNA expression in EBV-positive cell lines with latency I program (SavI, KemI, MutuI) or latency III program (SavIII, KemIII, MutuIII). Results are the mean ± standard error of the triplicates. ***, $P < 0.001$; ****, $P < 0.0001$.

that the EBV latent antigens besides EBNA1 played a critical role in upregulating KLF14 mRNA expression (Fig. 5C).

EBV latent antigens cooperate with the E2F-Rb-HDAC complex to target genes. In addition to *KLF10* and *KLF14* genes, we further detected the enrichment of the E2F-Rb-HDAC complex on other gene regions of crucial cellular factors in EBV-transformed LCL cells. These results identified the binding regions of the EBV latent antigens (EBNA1, EBNA2, EBNA3C, EBNA-LP) and the E2F-Rb-HDAC complex on *BCL2* and *MIR155HG* loci in LCLs (Fig. 6A and B). These two genes are representative key players that are induced in EBV-mediated lymphomagenesis (69, 70). More interestingly, although these viral antigens and cellular factors could bind to the coding regions of *BCL2* and *MIR155HG*, their binding regions were different although adjacent. Previous studies also showed these loci were highly active through interaction with other regions or transcription factors using RNAPII ChIA-PET and CTCF ChIA-PET data in the context of EBV infection (27, 28). Our findings further demonstrate that the E2F-Rb-HDAC complex may also involve complicated regulation as an important component of super-enhancers. In addition, the lack of EBNA1 enrichment on these genes indicated that it may not associate with the E2F-Rb-HDAC complex, which was consistent with

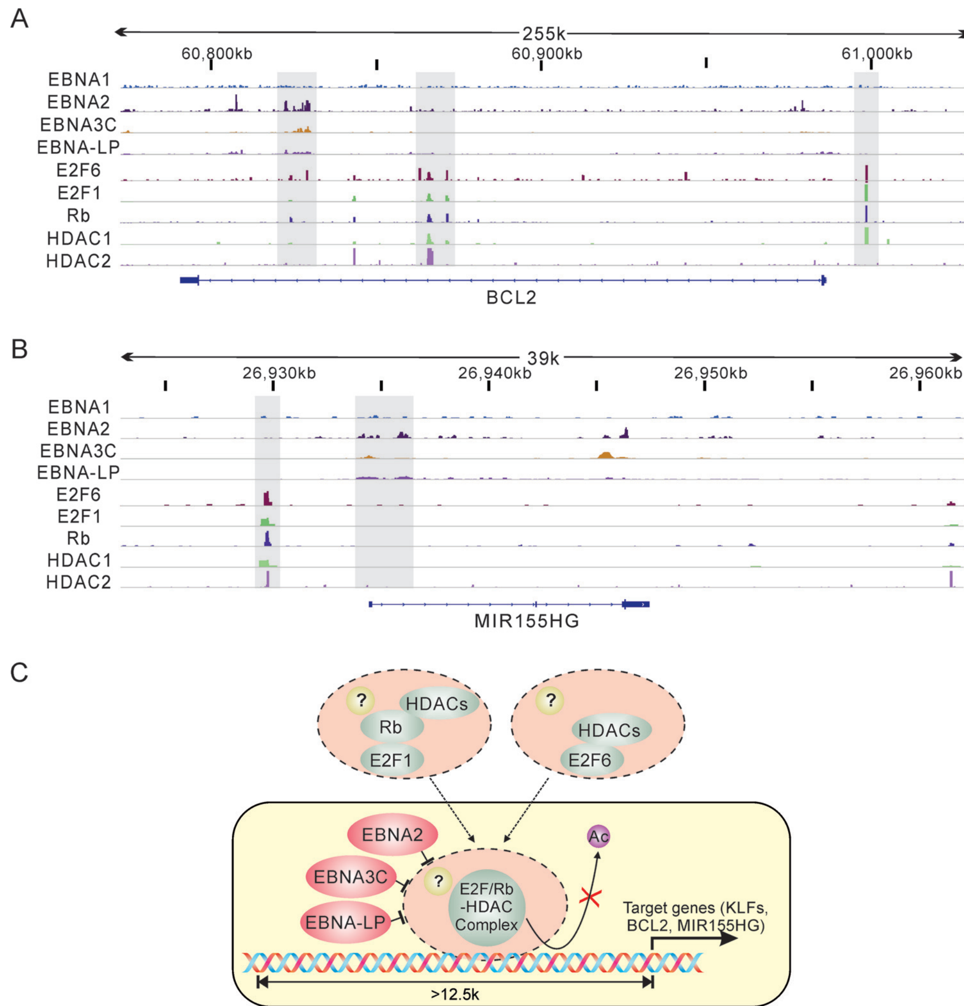

FIG 6 EBV latent antigens cooperate with the E2F-Rb-HDAC complex to target genes. (A and B) ChIP-Seq analysis shows the snapshot signal maps of representative targets *BCL2* (A) and *MIR155HG* (B) by the E2F-Rb-HDAC complex and EBV latent antigens. The peaks or binding regions are highlighted. (C) Models of human-EBV regulatory interactions in LCLs. E2F6 or E2F1/Rb cooperates with HDAC1 or HDAC2 and binds to the target enhancers as a complex to modulate the downstream gene expression (e.g., *KLF10/KLF14*, *BCL2*, *MIR155HG*), but EBV latent antigens (EBNA2, EBNA3C, and EBNA-LP) can reverse the suppressive activities and facilitate gene expression in EBV-transformed LCLs.

our previous conclusion on KLF14 expression in EBV-infected cells (Fig. 5B and C). These results indicate that EBV latent antigens can modulate the E2F-Rb-HDAC complex by regulating E2F6 or E2F1/Rb to further target the downstream gene expression pathways (Fig. 6C).

DISCUSSION

EBV is the first human tumor virus that was identified more than 50 years ago. EBV is best known for its ability to establish latent infection and transforming normal B-lymphocytes in the infected host. However, it is still a huge challenge to conditionally control the transformation process and fully describe the critical processes during EBV-induced tumorigenesis. A dysregulated transcriptional network is one of the hallmarks of EBV-induced transformation (71). With the rapid development of high-throughput sequencing methods, our current studies now provide a more comprehensive view of EBV antigens associated with cellular transcription regulatory networks (27, 28). Using chromatin immunoprecipitation and sequencing (ChIP-Seq), we identified the binding sites of several crucial cellular factors (E2F6, E2F1, Rb, HDAC, and HDAC2) and explored the patterns of EBV

induced regulation through long-range enhancers. The shared characteristics of their binding sites suggest analogous functions of these cellular factors. E2F1 is the transcriptional activator of E2F family members, while E2F6 is a repressor whose functions may be associated with the recruitment of the polycomb transcriptional repressor complex (72). Both E2F1 and E2F6 can bind to the E2F site within the promoters of numerous genes, which have also been shown in our results. Together with Rb, E2F1 binds to specific sites or regions of the human genome and recruits histone deacetylases (HDACs) or histone acetyltransferases (HATs) to modify chromatin structure (44, 73, 74). E2F6 can function in an Rb-independent pathway (1, 57). Thus, their similar mechanisms, in turn, are supported by their similar distribution on the human genome. In this study, we showed that KLF10 and KL14 were two new targets of the E2F-Rb-HDAC complex, which can be modulated by multiple EBV latent antigens. Our ChIP experiments showed that these cellular factors bound to the coding regions of KLF10 and KLF14 loci, but whether they can directly target their promoter regions need further investigation. More importantly, it is still not clear how EBV latent antigens upregulate KLF10 and KLF14 expression by modulating the E2F-Rb-HDAC complex and what are the key functions of KLFs in EBV-mediated lymphomagenesis.

Although E2F-Rb-HDAC could function as a complex to regulate the expression of target genes, the dynamic components of this functional structure are still unanswered. Rb interacts with other cellular proteins to remodel chromatin structure, including hBRM, BRG1, and SUV39H1 (75). To be specific, hBRM and BRG1 are the human homologs of SWI2/SNF2 that are the ATPases in yeast (76). These ATPases are involved in ATP-dependent nucleosome remodeling complexes to remodel chromatin structure and further control transcriptional repression and activation (13, 77). Rb also forms a repressor with HDACs and hSWI/SNF complex to suppress cyclin E and cyclin A expression (13). Moreover, Rb can inhibit E2F1 transcription activity by recruiting hBRM (78). The fact that Rb interacts with hBRM and E2F1 simultaneously suggests that the potential regulatory complex may cooperate at promoters with E2F binding sites (78). Loss of Rb function induces a p53-dependent apoptotic pathway, which may release E2F expression as well as ARF activation (79, 80). Besides Rb and hBRM proteins, p300/CBP and pCAF also act as a coactivator of E2F1 (81). Therefore, it is a huge challenge to completely understand how these cellular factors cooperate and organize on the human genome. The complex cooperation is likely to be critical for revealing the transcriptional regulatory networks in oncogenesis. Here, we concentrated on several key transcription factors (E2F6, E2F1, Rb, HDAC1, and HDAC2) to explore their genome-wide functional characteristics by using high-throughput sequencing strategies. The ChIP-Seq results showed that BCL2 and MIR155HG were two crucial targets of the E2F-Rb-HDAC complex. Previous studies also indicated the *BCL2* and *MIR155HG* loci were highly active and linked to EBV super-enhancers by cooperating with multiple transcription factors (27, 28). These results suggested that the E2F-Rb-HDAC complex together with EBV latent antigens were also involved in complex transcriptional regulation, but how this complex interacted with other transcription factors in the presence of EBV latent antigen is still a mystery.

The transcriptional regulatory network of human cells involves the modification of chromatin structure. In EBV-infected cells, how the latent antigens reorganize these complexes and reprogram transcription networks to facilitate tumorigenesis is also a complicated topic. Fortunately, many recent studies have implicated these interaction networks or models between EBV latent antigens and cellular factors by utilizing high-throughput genome sequence analysis (19). The novel proximity-based labeling techniques, such as BioID, APEX, and TurboID, have been developed as powerful approaches to capture the weak and transient interactions and map various scales of protein-protein interaction networks (82–84). How EBV latent antigens regulate host transcriptional networks to mediate lymphomagenesis will be further investigated with a combination of technical platforms for the development of novel treatments. Nevertheless, this study has demonstrated the potential functions of E2F-Rb-HDAC, which

involve super-enhancers, and has now identified two new KLF targets that can be promoted by EBV latent antigens and may be potential therapeutic targets for treating EBV-associated lymphomas.

MATERIALS AND METHODS

Cells and antibodies. EBV-transformed immortalized LCL1 (lymphoblastoid cell line) cells were generated in our laboratory and were grown in RPMI 1640 medium (HyClone, Logan, UT) supplemented with 5% fetal bovine serum (FBS), 25 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (85). Two Burkitt's lymphoma cell lines (BJAB and Raji), EBV converted BL41/B95.8 lymphoma cell line, and other EBV-positive cell lines (SavI, SavIII; KemI, KemIII; MutuI, MutuIII) were also cultured with the above-described RPMI 1640 medium.

The following antibodies were used for ChIP-Seq experiments: rabbit anti-E2F1 (sc-193X; Santa Cruz), rabbit anti-E2F6 (sc-22823X; Santa Cruz), mouse anti-Rb (G3-245; BD Pharmingen), rabbit anti-HDAC1 (ab7028; Abcam), rabbit anti-HDAC2 (ab7029; Abcam), normal mouse IgG (sc-2025; Santa Cruz), and normal rabbit IgG (sc-2027; Santa Cruz).

Chromatin immunoprecipitation (ChIP). The method has been described in the previous paper (86). Briefly, 30 million BJAB or LCL1 cells were cross-linked with 1% formaldehyde and harvested for sonication. Then cell lysates including sheared genomic DNA were immunoprecipitated with the indicated antibody (anti-E2F1, anti-E2F6, anti-Rb, anti-HDAC1, and anti-HDAC2) or normal IgG to collect the DNA fragments that can bind with the proteins of interest. The immune complexes were incubated with the salmon sperm DNA/protein A agarose slurry. Then the collected DNA was purified and subjected to real-time PCR analysis. The primers used for KLF10 were 5′-CCTTCCAGCCTCCATATTC-3′ and 5′-CAA CACAGGTAGCACAGAT-3′; those used for KLF14 were 5′-GACTTGTAATAGGCTTTGGTG-3′ and 5′-GGAGG AGGTCTGTCACAC-3′.

ChIP-Seq assay. The DNA library was prepared with a TruSeq ChIP library preparation kit (IP-202-1012; Illumina, San Diego, CA). Then the purified DNAs were sequenced using the Illumina HiSeq platform by the Genome Technology Access Center (GTAC) at the Washington University in St. Louis. These ChIP-Seq data are available at the NCBI Gene Expression Omnibus (GEO) with accession number GSE148165.

ChIP-Seq analysis. ChIP-Seq reads were mapped to the hg19 genome using Bowtie (87). Peak calling, the annotations of enriched regions, motif discovery, and pathway analysis were performed with HOMER (Hypergeometric Optimization of Motif EnRichment; http://homer.ucsd.edu/homer/) (48) or CLC Genomics Workbench version 12.0 (CLC Bio, Qiagen, USA). Pathway analysis was also performed using HOMER or Ingenuity Pathway Analysis (IPA; Qiagen, USA). The analyzed data were visualized using the R program (https://www.r-project.org/) and the Integrative Genomics Viewer (IGV) (88).

Quantitative real-time PCR. Total RNAs from the cells were extracted with TRIzol reagent (Invitrogen, Inc., Carlsbad, CA), treated with DNase I (Invitrogen, Inc., Carlsbad, CA), and reverse-transcribed with a Superscript II reverse transcriptase kit (Invitrogen, Inc., Carlsbad, CA). Then quantitative real-time PCR analysis was performed with the SYBR green real-time master mix (MJ Research, Inc., Waltham, MA) according to the manufacturer's protocol. The primers used for KL14 were 5′-AAGCCTATTACAAGTCGT C-3′ and 5′-TAAACTTCTTGTCGCAGTC-3′. GAPDH was set as an internal control as previously described (86). These assays were conducted in triplicate.

Statistical analysis. GraphPad Prism software version 6.01 was used for statistical analysis. The mean values with standard deviation (SD) were presented in this study, and the significance of differences was calculated with a 2-tailed Student's *t* test. The P value of <0.05 was considered statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Data availability. The EBV latent antigen-associated ChIP-Seq data in EBV-positive cells were downloaded from NCBI GEO data sets with accession numbers GSE73887 (EBNA1), GSE29498 (EBNA2), GSE52632 (EBNA3C), and GSE49338 (EBNA-LP). These RNA-Seq data and the histone marker-related ChIP-Seq data in GM12878 (EBV-transformed lymphoblastoid cell line) were analyzed and visualized with the WashU Epigenome Browser (https://epigenomegateway.wustl.edu/).

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