Hypoxia-inducible factor-1α plays roles in Epstein-Barr virus's natural life cycle and tumorigenesis by inducing lytic infection through direct binding to the immediate-early BZLF1 gene promoter


Abstract

When confronted with poor oxygenation, cells adapt by activating survival signaling pathways, including the oxygen-sensitive transcriptional regulators called hypoxia-inducible factor alphas (HIF-αs). We report here that HIF-1α also regulates the life cycle of Epstein-Barr virus (EBV). Incubation of EBV-positive gastric carcinoma AGS-Akata and SNU-719 and Burkitt lymphoma Sal and Kmll cell lines with a prolyl hydroxylase inhibitor, L-eminosine or deferoxamine, or the NEDDylation inhibitor MLN4924 promoted rapid and sustained accumulation of both HIF-1α and lytic EBV antigens. ShRNA knockdown of HIF-1α significantly reduced deferoxamine-mediated lytic reactivation. HIF-1α directly bound the promoter of the EBV primary latent-lytic switch BZLF1 gene, Zp, activating transcription via a consensus hypoxia-response element (HRE) located at nt -83 through -76 relative to the transcription initiation site. HIF-1α did not activate transcription from the other EBV immediate-early gene, BRLF1. Importantly, expression of HIF-1α induced EBV lytic-gene expression in cells harboring wild-type EBV, but not in cells infected with variants containing base-pair substitution mutations within this HRE. Human oral keratinocyte (NOK) and gingival epithelial (hGET) cells induced to differentiate by incubation with either methyl cellulose or growth in organotypic culture accumulated both HIF-1α and Blimp-1α, another cellular factor implicated in lytic reactivation. HIF-1α activity also accumulated along with Blimp-1α during B-cell differentiation into plasma cells. Furthermore, most BZLF1-expressing cells observed in lymphomas induced by EBV in NSG mice with a humanized immune system were located distal to blood vessels in hypoxic regions of the tumors. Thus, we conclude that HIF-1α plays central roles in both EBV's natural life cycle and EBV-associated tumorigenesis. We propose that drugs that induce HIF-1α protein accumulation are good candidates for development of a lytic-induction therapy for treating some EBV-associated malignancies.

Author summary

Most adults throughout the world are infected with Epstein-Barr Virus (EBV), a human herpesvirus frequently associated in a latent state with some cancers of epithelial and B-cell origin such as nasopharyngeal carcinoma and Burkitt lymphoma, respectively. To develop an oncolytic therapy for treating patients with EBV-associated cancers, we need a method to efficiently induce synthesis of lytic EBV proteins. The EBV protein encoded by its immediate-early BZLF1 gene usually mediates the switch into lytic viral infection. We show here that HIF-1α, a cellular transcription factor that accumulates in cells when deprived of normal levels of oxygen, can induce lytic EBV infection. HIF-1α mediates this switch by directly binding to a specific sequence located within the BZLF1 gene promoter, activating its expression. Importantly, we also show that deferoxamine, an FDA-approved drug that inhibits degradation of HIF-1α, can induce synthesis of lytic EBV proteins in some EBV-positive epithelial and lymphocytic cell lines. These findings indicate that HIF-1α-stabilizing drugs, administered in combination with nucleoside analogues such as ganciclovir, may be helpful as part of a lytic-induction therapy for treating some patients with EBV-positive malignancies.
Introduction

Epstein-Barr virus (EBV) is a ubiquitous human gamma herpesvirus that infects over 90% of the world’s population. In healthy hosts, primary infection after childhood often results in infectious mononucleosis (IM). Following primary infection, EBV establishes a life-long latent infection in a tiny subset of its host’s memory B cells where its genome is maintained as an episome that replicates in synchrony with the host’s cellular DNA (reviewed in [1,2]). Latency is characterized by expression of, at most, a small number of viral protein-encoding genes (EBNAs and LMPs), two non-coding RNAs (EBERs), and some micro (mi) RNAs (reviewed in [3]). Latent EBV infection is associated with some malignancies in humans, including nasopharyngeal carcinoma (NPC), some gastric cancers (GC), a subset of Burkitt lymphomas (BL), diffuse large B-cell lymphomas (DLBCL), and post-transplant lymphoproliferative diseases (PTLD) (reviewed in [1,4,5]). Several EBV-encoded latency proteins and miRNAs have been shown to contribute to cell transformation and tumorigenesis [1,3].

Like other herpesviruses, EBV’s long-term success requires it to undergo lytic as well as latent modes of infection during its life cycle. While latent infection permits persistence of the virus for the life of the host, lytic replication enables production of infectious virus necessary for transmission from cell to host and host to host. Thus, EBV occasionally reactivates out of latent infected B cells. Physiological inducers of EBV reactivation include B-cell antigen receptor (BCR) activation leading to plasma cell differentiation [2], butyrate [6,7], and transforming growth factor β (TGF-β) [8,9]. Subsequently, EBV infects differentiated cells within the normal oropharyngeal epithelium where infection is usually lytic [1,2,10].

EBV reactivation is initiated by transcriptional activation of one or both of the viral immediate-early (IE) gene promoters, Zp and Rp, leading to production of its two IE proteins, Zta (the product of the BZLF1 gene; also called Z, ZEBRA, and EB1) and Rta (the product of the BRLF1 gene; also called R), respectively. Synthesis of Zta is sufficient to induce reactivation in most EBV-positive (EBV⁺) cell lines [11], while Rta induces reactivation in some cell lines [12,13]. Rta and Zta are transcription factors that then activate each other’s promoters [12,14,15] and, subsequently, activate expression of EBV’s early (E) genes, including BMRF1, a viral DNA polymerase processivity factor [also called early-antigen diffuse (EAD)], and BGLF4, a virus-encoded protein kinase (reviewed in [16]).

Given that expression of the BZLF1 gene serves as the primary gatekeeper to the viral latent-to-lytic switch in most EBV⁺ cell lines, transcriptional regulation of Zp has been studied extensively. Numerous cis-acting elements and their cognate trans-acting factors have been identified that contribute to silencing during latency and activation in response to inducers (reviewed in [16]).

Poor oxygenation, i.e., hypoxia, contributes to tumor progression and resistance to conventional chemotherapy (reviewed in [17–19]). The mechanisms by which cells respond to hypoxic environments are known (reviewed in [20,21]). Under normal oxygen tension corresponding to approximately 21% O₂, cellular transcription factors called hypoxia-inducible factor alphas (HIF-αs) are synthesized but rapidly degraded via the ubiquitin-dependent proteasome pathway. Three distinct genes encode the HIF-αs (HIF-1α, HIF-2α, and HIF-3α). Hydroxylation of specific proline residues by oxygen-dependent cellular prolyl hydroxylases (e.g., PHD2, encoded by the EGLN1 gene) marks these proteins for ubiquitin-mediated degradation. The hydroxylation reaction catalyzed by PHDs also involves the conversion of α–ketoglutarate to succinate, Fe⁺⁺ to Fe³⁺, and O₂ to CO₂, with vitamin C required for the regeneration of Fe²⁺. Under hypoxic conditions (or in the presence of iron chelators or competitors), PHDs fail to hydroxylate HIF-αs, resulting in accumulation of these proteins to high levels. Stabilized HIF-αs form heterodimers with their constitutively present HIF-βs, thereby activating transcription of downstream hypoxia-responsive elements (HREs) related to angiogenesis, anaerobic metabolism, and erythropoiesis.

The roles hypoxia and HIF-1α play in the life cycle of Kaposi’s sarcoma herpesvirus (KSHV), another member of the gamma herpesvirus family, have been extensively studied (reviewed in [22,23]). Analogously, Jiang et al. [24] reported that incubation of the EBV⁺ marmoset-derived B-cell line, B95-8, in 2% oxygen conditions leads to induction of Zta synthesis within one-to-two days, and Murata et al. [25] confirmed that hypoxia (1% oxygen; 36 h) induces BZLF1 gene expression in human EBV⁺ Akata B cells and LCLs as well as B95-8 cells.

Here, we report that drugs that mimick hypoxia induce lytic EBV infection in some EBV⁺ epithelial and B-cell lines by a HIF-1α-dependent mechanism. HIF-1α induces the switch to lytic-gene expression through directly activating BZLF1 gene expression by sequence-specific binding to an HRE located within Zp. We further show that HIF-1α can play important roles in EBV’s natural life cycle and tumorigenesis induced by this virus. These findings suggest a new class of drugs that may be useful in the development of a lytic-induction therapy for treating patients with some EBV-associated malignancies.

Results

HIF-α stabilizers induce EBV reactivation in some EBV⁺ cell lines

Our long-term objective is to find drugs suitable for use in EBV-targeted oncolytic therapy [26,27]. Thus, we chose to mimick hypoxia by incubating cells with deferoxamine (DFO; also called Desferal) or L-mimosine (Mim; also called Leucenol), two drugs that inhibit prolyl hydroxylase activity by chelating iron [28]. The EBV⁺ cell lines examined were Burkitt lymphoma-derived Sal and KemmII and gastric carcinoma-derived AGS-Akata and SNU-719. SNU-719, Sal, and KemmII retain their original-infecting EBV genomes. SNU-719 cells have type I latency plus LPM2A, Sal cells have Wp-restricted latency, and KemmII have type III latency. In an initial experiment, we found that incubation of Sal cells with mimosine promoted both stabilization of HIF-1α and induction of synthesis of the immediate-early (IE) lytic EBV antigen, Zta (Fig 1A). However, because mimosine is not FDA-approved for internal use, we largely focused on DFO in subsequent experiments. Incubation of all four of these cell lines with DFO for 24 h promoted stabilization of HIF-1α protein along with inducing synthesis of Zta (Fig 1B–1E). Quantification of the efficiency of EBV reactivation by
Hypoxia-inducible factor-1α plays roles in Epstein-Barr virus’s natural life cycle and tumorigenesis by inducing lytic infection through direct binding.

DFO efficiently induces EBV reactivation in a subset of HIF-1α expressed in cell types of physiological relevance to EBV. Given this finding, most of the studies presented here were performed with HIF-1α. We occasionally confirmed our findings with HIF-2α and did not conduct further studies with HIF-3α.

Thus, although HIF-2α may contribute to EBV's life cycle under some conditions in epithelial cells, HIF-1α appears to be the primary human B-cells throughout the various stages of B-cell differentiation into plasma cells.

DFO efficiently induces EBV reactivation in a subset of HIF-1α cells

If HIF-1α induces EBV reactivation, one would expect most Zta+ cells to also be HIF-1α+. To determine the level of coincidence between Zta+ and HIF-1α+ cells, we performed dual immunofluorescence staining (IFS) assays. Consistent with our hypothesis, we found that almost all of the Zta+ cells were also HIF-1α+ in AGS-Akata cells that had been incubated with DFO for 24 h (Fig 2).
Hypoxia-inducible factor-1α plays roles in Epstein-Barr virus’s natural life cycle and tumorigenesis by inducing lytic infection through direct binding of the viral transactivator Zta. HIF-1α-negative cell we observed was likely the consequence of AGS-Akata cells exhibiting some spontaneous reactivation (e.g., S1B Fig). Thus, we conclude that DFO efficiently induces EBV reactivation in AGS-Akata cells, at least in part, by stabilizing HIF-1α.

Fig 2. Dual immunofluorescence staining indicates DFO efficiently induces synthesis of Zta protein in a subset of HIF-1α-expressing EBV-positive cells.
AGS-Akata cells grown on cover slips were incubated for 24 h in the absence (-) or presence (+) of 200 μM DFO prior to fixing and processing for co-detection by IFS of the proteins Zta (green) and HIF-1α (red). DFO-treated cells were independently probed with the green-conjugated secondary antibody absent primary antibodies to control for background GFP encoded by the virus.

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DFO induction of Zta synthesis is mediated primarily via HIF-1α

To demonstrate a direct causal role of HIF-1α in reactivation, we evaluated induction of synthesis of lytic EBV antigens after addition of HIF-1α. AGS-Akata cells were co-transfected with: (i) a plasmid expressing an oxygen-insensitive variant of HIF-1α that contains alanine substitutions in the proline residues targeted for hydroxylation by PHDs; and (ii) a plasmid expressing HIF-1α’s heterodimeric partner, HIF-1β/ARNT. Addition of HIF-1α/HIF-1β was sufficient to strongly induce synthesis of Zta and EAD (Fig 3A).

Fig 3. HIF-1α addition is sufficient to induce EBV reactivation and necessary for efficient induction by DFO.
(A) Immunoblots showing addition of HIF-1α is sufficient to induce lytic EBV reactivation. AGS-Akata cells grown in 10-cm dishes were transfected with 1 μg each of pHA-HIF-1α P402A/P564A-pcDNA3 plus pHIF-β (+) or 2 μg of their empty vector, pcDNA3, as a control (-) and incubated for 48 h prior to preparation of whole-cell extracts. Data are representative of numerous independent experiments. (B) Immunoblots showing knockdown of HIF-1α inhibits DFO-induced synthesis of EBV lytic antigens. Lanes 1–6, AGS-Akata cells maintained in 10-cm dishes were co-transfected with 0.8 μg of each of five lentiviruses that express different shRNAs targeting HIF-1α (lanes 5–6) or 4 μg of a lentivirus that expresses the non-targeting shRNA cntl. #1 or cntl. #2 (lanes 1–2 and lanes 3–4, respectively). Two days later, the cells were incubated in the absence (-) or presence (+) of 200 μM DFO for 24 h prior to harvesting and preparation of whole-cell extracts. Lanes 7–10, Sal cells were infected with the indicated packaged lentiviruses; three days later, the cells were incubated in the absence (-) or presence (+) of 200 μM DFO for 24 h and processed likewise. Data are representative of two independent experiments. GADPH served as a loading control.

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We also performed a reciprocal experiment. Knockdown of HIF-1α expression by 80%-90% in AGS-Akata cells resulted in a comparable level of loss of DFO-induced synthesis of Zta and EAD (Fig 3B, lanes 1–6). Similar findings were observed in Sal cells infected with these lentiviruses (Fig 3B, lanes 7–10). Thus, we conclude that DFO-directed induction of lytic EBV infection is mediated largely by HIF-1α.

HIF-1α primarily induces EBV lytic-gene expression by activating transcription from Zp

HIF-1α induces KSHV reactivation by directly enhancing expression of its ORF50 gene, the orthologue of EBV’s BRLF1 gene [32,33]. Thus, we asked whether HIF-1α reactivates EBV by inducing transcription from Rp and/or Zp. HEK 293T cells were transiently co-transfected with plasmids expressing the oxygen-insensitive variant of HIF-1α, HIF-1β, and reporters driving
Hypoxia-inducible factor-1α plays roles in Epstein-Barr virus’s natural life cycle and tumorigenesis by inducing lytic infection through direct binding of enhancer regions.

Luciferase expression from Rp or Zp. We used an Rta expression plasmid as a positive control since Rta is a potent transcriptional activator of both Zp and Rp [15]. While addition of HIF-1α/HIF-1β activated transcription from the Zp-luc reporter approximately 24-fold, it activated the Rp-luc reporter similarly to the four-fold activation observed with the negative control TATA-luc reporter (Fig 4). As expected, Rta robustly activated both reporters. Thus, HIF-1α/HIF-1β heterodimers activate transcription from Zp approximately six-fold above the non-specific level observed in this assay while failing to activate specifically transcription from Rp. Thus, in contrast to KSHV, we conclude that HIF-1α regulates lytic EBV infection by activating expression of the BZLF1 gene, not the BRLF1 gene.

Fig 4. HIF-1α induces transcriptional activation from Zp, but not Rp.
293T cells maintained in 24-well plates were co-transfected with (i) 200 ng DNA of a pGL3-Basic luciferase reporter containing the nt -30 to +30 region of the HSV TK gene (pTATA-luc) as a control, the nt -221 to +30 region of Zp (pWTZp-luc), or the nt -1069 to +38 region of Rp (pWTRp-luc), and (ii) pHA-HIF-1αP402A/P564A-pcDNA3 plus pHIF-1β (40 ng each), pcDNA3-BRLF1 (30 ng) as a positive control, or pcDNA3 (80 ng) as a negative control. Cells were harvested 48 h later, and luciferase activities were determined. Data obtained with each reporter were normalized to the value obtained when co-transfected with pcDNA3; they are averages from three independent experiments each performed in triplicate; error bars indicate standard errors of the mean. **, p < 0.01.

HIF-1α directly activates transcription from Zp via an HRE
To determine how HIF-1α activates BZLF1 gene expression, we performed an in silico analysis of Zp and noted a single consensus HRE located from nt -83 through -76 relative to the Zp transcriptional initiation site (Fig 5A). To examine whether HIF-1α-dependent transactivation of Zp mapped to this sequence, we constructed a set of base-pair substitution mutant variants of our WT luciferase reporter, pWTZp-luc (Fig 5B). These mutations were designed to avoid disrupting bases that overlap the adjacent ZIIR silencing element [34, 35]. Reporter assays performed with these variants of pZp-luc showed that the WT and ZIIR mutant promoters were activated by HIF-1α/HIF-1β approximately five- to eight-fold above the non-specific activation observed with the minimal TATA box-containing control promoter while none of the 3-bp substitution mutants in the putative HRE were activated above this non-specific level (Fig 5C). Even the 1-bp substitution mutation present in mutant M1 significantly reduced activation by HIF-1α/HIF-1β. Analysis of the basal activity of these mutants in the absence of HIF-1α and of a non-overlapping mutant only altered in nt -77 and -76 of the Zp HRE ruled out the possibility that these HRE mutations were affecting binding of a repressor (S2 Fig). Similar results were obtained when we used an expression plasmid that encodes an oxygen-insensitive variant of HIF-2α in place of the HIF-1α one (Fig 5D). Thus, we conclude that Zp contains a transcriptionally functional HRE that includes nt -79 through -81.
Hypoxia-inducible factor-1α plays roles in Epstein-Barr virus’s natural life cycle and tumorigenesis by inducing lytic infection through direct binding to the Zp HRE.

HIF-1α binds the Zp HRE

HREs act as sequence-specific binding elements for HIF-α/β heterodimeric complexes. To demonstrate that HIF-1α/HIF-1β heterodimers bind to the Zp HRE, we performed in vitro DNA-binding assays. Our protein source of HIF-1α/HIF-1β complexes was nuclear extract obtained from EBV-negative AGS cells incubated for 24 h with 200 μM CoCl₂, an iron competitor. A radiolabeled, double-stranded oligonucleotide containing a consensus HRE sequence, 5’-CACGTC-3’, served as probe (Fig 6C, HRE WT). We identified the HIF-1α-containing protein-DNA complex by showing it was lost by incubation with a HIF-1α-specific antibody (Fig 6A). Competition electrophoretic-mobility-shift assays (EMSAs) were performed by pre-incubation of the extract with various amounts of the unlabeled, double-stranded WT or mutant (MT) oligonucleotides indicated in panel C. WT Zp HRE-containing oligonucleotide competed for binding the HIF-1α/HIF-1β complex as well as the consensus WT HRE oligonucleotide (Fig 6B, lanes 9–11 vs. lanes 3–5, respectively) while the 3-bp mutant variant of this consensus HRE oligonucleotide failed to compete (Fig 6B, lanes 6–8 vs. lane 2). Likewise, a 3-bp mutant variant of the Zp HRE-containing oligonucleotide corresponding to the M3 mutation that abolished HIF-1α/HIF-1β-dependent transcriptional activation of Zp-luc (Fig 5) also largely failed to compete for binding HIF-1α/HIF-1β complexes (Fig 6B, lanes 12–14 vs. lane 2). Thus, the trans-activation and DNA-binding activities of HIF-1α co-localize to the HRE present within Zp.

HIF-1α binds Zp in vivo

We next performed ChIP assays to show HIF-1α binds Zp in the physiological context of whole EBV genomes. SNU-719 and Sal cells incubated (+) or not (-) with 200 μM DFO for 24 h served as the source of chromatin given this treatment induces abundant accumulation of HIF-1α in these cells (Fig 7A). Quantitative PCR analysis of these samples following chromatin precipitation with HIF-1α-specific versus IgG control antibody indicated that this HIF-1α-specific antibody precipitated Zp approximately four-fold...
Hypoxia-inducible factor-1α plays roles in Epstein-Barr virus’s natural life cycle and tumorigenesis by inducing lytic infection through direct binding to Zp, an HIF-α-responsive enhancer. We observed that HIF-1α RNA is more abundant than HIF-2α RNA in all of the EBV-infected cell lines we have examined to date. Nevertheless, given our finding that HIF-2α can also activate Zp in reporter assays, we conclude that HIF-1α-induced reactivation of EBV requires the Zp HRE.

To confirm that HIF-1α induction of lytic EBV infection truly occurs via binding to this Zp HRE rather than indirectly via downstream signaling events, we constructed two independent HRE variants of EBV containing the M2 and M4 substitution mutations analyzed in our reporter assay (Fig 5) within the context of the p2089 BAC [36]. 293T cells were transfected in parallel with these two EBV HRE mutant BACs alongside their parental WT EBV BAC and selected for resistance to hygromycin to establish the cell lines 293T-EBV M2, 293T-EBV M4, and 293T-EBV WT, respectively. Confirming our observation with AGS-Akata cells (Fig 3A), co-transfection of 293T EBV-WT cells with plasmids expressing the oxygen-insensitive variant of HIF-1α along with HIF-1β efficiently induced expression of EBV IE and E genes (Fig 8A, lane 2 vs. lane 1). Strikingly, co-transfection of HIF-1α/HIF-1β expression plasmids into 293T cells latently infected with either the M2 or M4 HRE mutant variant of EBV failed to induce synthesis of lytic EBV antigens above the background level of spontaneous reactivation (Fig 8A, lane 4 and lane 6, respectively).
HIF-1α protein accumulates during epithelial and B-cell differentiation

Why might EBV have evolved to contain an HRE within Zp? To answer this question, we examined whether the appearance of HIF-1α protein during differentiation of normal epithelial and B cells coincides with the cell types in which lytic EBV infection takes place. In B cells, lytic EBV reactivation occurs when memory B-cells begin to differentiate into plasma cells [2]. To determine when functionally active HIF-1α protein is present in B cells, we mined existing microarray data sets obtained from B cells harvested at eight different stages of differentiation, ranging from naïve B cells to fully differentiated plasma cells (Fig 9). HIF-1α mRNA is present at high levels in all of these stages, declining somewhat only during the very last stage. However, functionally active HIF-1α protein, as measured by expression of the HIF-1α-activated genes VEGFA and PDK1, dramatically increases in the post-memory cell preplasmablast and plasmablast stages, respectively. These are the same stages during B-cell differentiation when expression of both ZEBs plummets (possibly due, in part, to HIF-1α also activating synthesis of miR-429 [37], a down-regulator of ZEB levels [38,39]), and expression of Blimp-1α and XBP-1 dramatically increases. Thus, the stages during B-cell differentiation when EBV reactivates are coincident with the stages when three of the Zp activators (HIF-1α, Blimp-1α [40], and XBP-1s [41,42]) appear and two of the major Zp repressors (ZEB1 and ZEB2 [43,44]) disappear.

Another stage of EBV’s natural life cycle involves the infection of differentiated epithelial cells by EBV (either free virions or virus produced in reactivated EBV+ B cells) [10]. Expression of Blimp-1α is also induced during epithelial cell differentiation, synergizing with KLF4 to activate transcription from both Zp and Rp [40,45]. To determine whether HIF-α protein accumulation is induced by epithelial cell differentiation, we incubated telomerase (TERT)-immortalized human normal oral keratinocyte (NOK) cells with the differentiation-inducing agent, methylcellulose (MC) (Fig 10A). Both HIF-1α and HIF-2α protein, along with some Blimp-1α, appeared within 2 h of MC addition; they remained present for at least 12 h. Thus, their stabilization may be among the earliest events to occur during epithelial cell differentiation, hours before the appearance of involucrin, another marker of epithelial cell differentiation. The kinetics of appearance of HIF-1α and Blimp-1α were similar in MC-treated NOK-Akata, cells infected with EBV (Fig 10B). This latter finding suggests that regulation of the stabilization of HIF-1α protein during epithelial cell differentiation occurs independently of the presence of EBV. We examined likewise hTERT-infected human gingival epithelial (hGET) cells. In this case, HIF-1α protein and Blimp-1α were both abundantly present, along with involucrin, 48 h after addition of MC (Fig 10C, lane 4). HIF-1α protein also accumulated together with Blimp-1α and involucrin when NOK cells were induced to differentiate by growth in organotypic culture (Fig 10D). Thus, both HIF-1α and Blimp-1α are present in differentiated cells of the types present in the human oral cavity.
HIF-1α plays roles in Epstein-Barr virus’s natural life cycle and tumorigenesis by inducing lytic infection through direct binding...
Hypoxia-inducible factor-1α plays roles in Epstein-Barr virus’s natural life cycle and tumorigenesis by inducing lytic infection through direct binding of HREs in Zp.

**Role of HIF-1α in EBV’s natural life cycle and tumorigenesis**

Primary human tumor cell lines, including primary tumors and cell lines we have examined to date. Consistent with this finding, the gastric cancer-derived cell lines, SNU-719 and AGS-Akata, were the only ones in which we detected HIF-2α protein upon incubation with DFO (Fig 1D). Previous reports of others likewise indicated preferential accumulation of HIF-1α protein with exposure to hypoxia in EBV+ LCLs that contain little HIF-2α mRNA [49] and in EBV+ NPC-derived cell lines that contain some HIF-2α mRNA [50]. Thus, we conclude that HIF-1α is the primary HIF-α of physiological relevance to EBV’s natural life cycle and in EBV+ tumors.

**HIF-1α versus HIF-2α**

We also presented evidence that HIF-1α was required for induction of EBV reactivation by the hypoxia mimic DFO (Fig 3B). Expression of an oxygen-insensitive variant of HIF-1α was also sufficient to induce EBV reactivation (Figs 3A and 8A). HIF-1α activated transcription from Zp, but not Rp, in both a reporter assay and in the context of whole EBV genomes (Figs 4 and 8A), with activation mediated by a consensus HRE located at nt -83 through -76 relative to the transcription initiation site of Zp (Figs 5 and 8). EMSAs and ChIP assays confirmed that HIF-1α bound Zp via this HRE (Figs 6 and 7). Remarkably, 3-bp substitution mutations in this HRE were sufficient to eliminate HIF-1α-mediated reactivation in the context of the intact viral genome (Fig 8). Thus, HIF-1α induces lytic EBV infection by sequence-specific binding to a single HRE located within Zp.

**HRE versus ZIIR elements of Zp**

The HRE identified here overlaps the previously identified ZIIR element of Zp [34, 35] (Fig 5B). Thus, one possibility was that binding of HIF-1α to this HRE activates transcription from Zp by displacing the yet-to-be-identified ZIIR repressor. Inconsistent with this hypothesis was our finding that mutations known to relieve ZIIR-mediated repression affected neither HIF-1α- nor HIF-2α-induced activation of transcription from Zp (Fig 5C and 5D, respectively) unless they also impinged upon the HRE element (S2 Fig). Furthermore, HRE mutations that abolished HIF-α-induced reactivation of EBV had no effect on the frequency of spontaneous reactivation (Fig 8), a frequency enhanced in ZIIR mutant variants of EBV [35]. Thus, we conclude that the HRE and ZIIR elements are genetically distinguishable, independently acting regulatory elements of Zp, with HIF-α proteins functioning as transcriptional activators via binding to the HRE.

**HIF-1α versus HIF-2α**

The sequence encompassing the HRE present in the promoter of KSHV’s latent gene, ORF73 [encoding latency-associated nuclear antigen (LANA)], is identical to that of the EBV Zp HRE we identified here, with both HREs being responsive to both major HIF-α isoforms [48] (Figs 5 and 8). However, HIF-1α was the predominant HIF-α expressed at the RNA level in all of the EBV+ primary tumors and cell lines we have examined to date. Consistent with this finding, the gastric cancer-derived cell lines, SNU-719 and AGS-Akata, were the only ones in which we detected HIF-2α protein upon incubation with DFO (Fig 1D). Previous reports of others likewise indicated preferential accumulation of HIF-1α protein with exposure to hypoxia in EBV+ LCLs that contain little HIF-2α mRNA [49] and in EBV+ NPC-derived cell lines that contain some HIF-2α mRNA [50]. Thus, we conclude that HIF-1α is the primary HIF-α of physiological relevance to EBV’s natural life cycle and in EBV+ tumors.

**Role of HIF-1α in EBV’s natural life cycle and tumorigenesis**

Much literature exists indicating HIF-1α plays central roles in regulating both lytic infection and tumorigenesis by KSHV (reviewed in [22,23]). Functional HREs are present within the promoter regions of KSHV’s latent gene, ORF73/LANA, as well as its IE lytic gene, ORF50/RTA, and lytic ORF34-ORF37 gene cluster [32,48,51]. HIF-1α complexes with LANA to activate ORF50 gene expression...
Hypoxia-inducible factor-1α plays roles in Epstein-Barr virus’s natural life cycle and tumorigenesis by inducing lytic infection through direct binding of HIF-1α and HIF-1α. Quite likely, one may be able to increase considerably the percentage of the EBV treatment with a stabilizer of HIF-1α may well be sufficient, reducing potential adverse reactions due to off-target effects of the drug activated by an inducer, Zta synthesis usually continues after the inducer is removed because of its positive feedback loop with Rta phosphorylate GCV (indicated by the presence of phosphorylated forms of EAD (expression of EBV early- and late-lytic genes (strategy to achieve efficient EBV lytic-induction therapy. Transient expression of HIF-1α induced sufficient Zta synthesis to promote (GCV), may be one way to achieve this goal (activated by KSHV, the relationship between EBV’s latent gene products and HIF-1α is also complex. EBNA3A and EBNA-LP bind PHDs, blocking their catalytic activity and, thereby, inhibiting oxygen-dependent degradation of HIF-1α [53]. LMP1 promotes accumulation of HIF-1α by signaling PHD1 and PHD2 degradation pathways [54,55]. EBNA3A stabilizes HIF-1α via protein-protein interactions [53], a complex somewhat analogous to the LANA/HIF-1α complex. However, these above-mentioned EBV-encoded proteins are clearly not necessary for HIF-1α-induced activation of BZLF1 gene expression given we showed here that HIF-1α can induce transcription from Zp in EBV-negative cells and Zta synthesis in EBV+ Sal cells that are in a Wp-restricted latency in which these proteins are not expressed. In latency types in which these above-mentioned proteins stabilize HIF-1α, other factors are likely also present in the cells to inhibit HIF-1α from inducing lytic reactivation. EBNA1, present in all EBV+ cells, also has been reported to enhance HIF-1α activity, most likely indirectly via its effects on AP-1 [56].

We propose that HIF-1α plays central roles in regulating both lytic replication and tumorigenesis by EBV. Regarding EBV’s natural life cycle, we hypothesize that B cells from the naïve B-cell through memory B-cell stages lack functional HIF-α activity as well as Blimp-1a and XBP-1s (other known inducers of BZLF1 gene expression), while containing several known direct or indirect repressors of this gene [9,43] and inhibitors of Zta activity [57,58] (Fig 13). Thus, EBV infection tends to go latent. However, if an EBV-infected B cell begins to undergo plasma cell differentiation, the virus may reactivate due to the appearance of functionally active HIF-1α along with these other activators and loss of these repressors. Likewise, when epithelial cells differentiate, they accumulate HIF-α along with Blimp-1a and KLF4 (known inducers of BRLF1 as well as BZFL1 gene expression [40,45]) and lose repressors of these genes such as the ZEBs (Fig 13). Thus, when EBV virion particles or EBV-infected B cells come into close contact with differentiated epithelial cells within the oral cavity, the introduction of EBV genomes into these cells can lead to lytic replication and production of infectious virus, helping to spread the virus from cell-to-cell and host-to-host.

How might HIF-1α and the Zp HRE contribute to tumorigenesis by EBV in vivo? We propose that HIF-αs contribute via two routes to tumor growth in EBV+ cancers. As is true of most tumors, EBV+ tumors develop hypoxic regions as they enlarge (e.g., Fig 11C, S3A Fig; [59–61]), leading to accumulation of the HIF-α whose genes are being expressed. These HIF-αs then activate expression of a variety of cellular genes involved in angiogenesis and anaerobic metabolism that help the tumor to continue to enlarge (reviewed in [62]). In the case of EBV+ tumors, presence of HIF-α also contributes to activation of BZLF1 gene expression, leading to EBV lytic-gene expression in some tumor cells. We showed here by examining EBV-induced lymphomas and EBV+ gastric cancer xenografts that Zta+ cells were preferentially located in regions of the tumors that were clearly hypoxic as indicated by Hypoxprobe staining or, presumably, hypoxic because they were located distal to blood vessels (Figs 11 and 12, S3–S5 Figs). Thus, we propose the following model: Hypoxic regions develop in EBV+ tumors as they grow in size, leading to accumulation of HIF-1α and, in some cases, HIF-2α. Prior to angiogenesis, HIF-α increases the frequency of lytic EBV infection in these hypoxic regions, with these lytic-infected cells secreting a variety of cellular and viral factors, some of which contribute to the enhancement of tumor growth (reviewed in [47]).

**HIF-1α and oncolytic therapy**

The goal of chemotherapy is to kill cancer cells while minimizing harm to healthy cells. Treatment of some EBV+ cancers with minimally toxic drugs that rapidly and efficiently induce EBV lytic-gene expression, in combination with prodrugs such as ganciclovir (GCV), may be one way to achieve this goal [16,63] and references cited therein). Based upon the findings presented here, we propose that briefly targeting the PHDs or other enzymes that regulate degradation of HIF-1α (e.g., NAE) may be useful as part of a strategy to achieve efficient EBV lytic-induction therapy. Transient expression of HIF-1α induced sufficient Zta synthesis to promote expression of EBV early- and late-lytic genes (Fig 1C). Furthermore, these expressed early-lytic genes included BGLF4 [as indicated by the presence of phosphorylated forms of EAD (e.g., Figs 3 and 8)], the gene that encodes the EBV-PK that can phosphorylate GCV [64]. Intrinsic features of EBV and HIF-1α make this strategy feasible: (i) Once BZLF1 gene expression is activated by an inducer, Zta synthesis usually continues after the inducer is removed because of its positive feedback loop with Rta (e.g., Fig 1C); and (ii) The HIF-αs are rapidly degraded once HIF-α-stabilizing drugs are removed (e.g., Fig 1C). Thus, brief treatment with a stabilizer of HIF-1α may well be sufficient, reducing potential adverse reactions due to off-target effects of the drug and HIF-1α. Quite likely, one may be able to increase considerably the percentage of the EBV+ cells reactivated by using DFO, MLN4924, or another HIF-1α stabilizer in combination with other drugs known to activate BZLF1 gene expression via different cellular signaling pathways (e.g., HDAC inhibitors).
We were fortunate to identify here an already FDA-approved drug as a possible candidate for use in lytic-induction therapy. DFO and the FDA-approved oral iron-chelators deferasirox and deferiprone, are used to treat iron overload and toxicity that result from frequent blood transfusions [65]. DFO-based therapy is also emerging as a tool for treating a variety of diseases, including persistent anemia, impaired angiogenesis resulting from diabetes mellitus, and numerous neurodegenerative disorders (reviewed in [66]).

Materials and methods

Ethics statement
The mouse experiments were approved by the UW-Madison Institutional Animal Care and Use Committee (protocol #M005197-A01) and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The mice were sacrificed by cervical dislocation under isoflurane anesthesia. The UW IRB classified the work with human tissues and cells as exempt.

Cells
Sal cells (a gift from Alan Rickinson via Bill Sugden) were derived from an EBV<sup>+</sup> BL; they are co-infected with wild-type and EBNA2-deleted EBV genomes and maintain a Wp-restricted latency [67]. KemIII cells (a gift from Alan Rickinson via Jeff Sample), derived from an EBV<sup>+</sup> BL, are currently in type III latency and express LMP1. These B-cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS and 100 units/ml penicillin and 100 μg/ml streptomycin (pen-strep; Life Technologies). SNU-719 cells (obtain from Jin-Pok Kim via Bill Sugden), derived from an EBV<sup>+</sup> gastric carcinoma, retain their original EBV genome [68]; they were maintained likewise. AGS-Akata cells, an EBV-infected clonal derivative of AGS cells (derived from a human gastric carcinoma; obtained from William Kaelin via Addgene (#18955 and #18956, respectively). A HIF-1β expression plasmid, pSV-Sport-ARNT [72], was obtained from Christopher Bradfield. Plasmid pZpWT-luc contains the nt -221 to +30 region of Zp relative to the transcription initiation site cloned between the KpnI and HindIII sites of the luciferase reporter plasmid, pGL3-Basic (Promega) [73]. The mutant variants of it shown in Fig 5B contain the indicated base pair substitution mutations; they were generated by Quick Change methodology (Stratagene), with pZpWT-luc serving as template and synthetic oligonucleotides containing the desired mutations surrounded by 10 bases of wild-type sequence serving as primers. Plasmid pWTRP-luc contains the nt -1069 to +38 region of Rp relative to the transcriptional initiation site cloned between the KpnI and HindIII sites of pGL3-Basic. Plasmid pTATA-luc [74] served as a negative control. Plasmids pSG5-BZLF1 and pRTS15 (kindly provided by Diane Hayward) express Zta and Rta, respectively, from the SV40 early promoter [75]. Plasmid pcDNA3-BRLF1 expresses Rta from the CMV IE promoter [76]. Plasmid p2089 (a generous gift from Wolfgang Hammerschmidt) is a BAC containing the entire genome of the B95.8 strain of EBV [36] or HRE mutant variants thereof were maintained in DMEM additionally supplemented with 100 μg/ml hygromycin B.

NOK (a gift from Karl Munger) are telomerase (hTERT)-immortalized normal oral keratinocyte (NOK) cells [13]. NOK-Akata, clone 2 (generously obtained from Bill Sugden), are NOK cells (with WT p53) that are latently infected with an Akata-GFP strain of EBV [13]. NOK (clones #1 and #3) cells are clonal isolates of NOK cells (with WT p53). These cell lines were maintained in an undifferentiated state by growth in keratinocyte serum-free medium (K-SFM; Life Technologies) supplemented with epidermal growth factor, bovine pituitary extract, pen-strep. The NOK-Akata growth medium also included 50 μg/ml G418.

hTERT-transduced human gingival epithelial (hGEP) cells were generated as follows. A frozen pool of primary human gingival epithelial cells (HGEPP) was obtained from CellnTEC. Upon thawing, the cells were initially grown in their specialty medium (CnT-PR; CellnTEC) and re-frozen. These cells were then passaged in K-SFM supplemented with a ROCK inhibitor (10 μM Y-27632 Di-HCl; Selleck Chemical #50-863-6) and infected with pBABE-puro-hTERT (Addgene plasmid #1771; a gift from Bob Weinberg) [70], a recombinant retrovirus expressing human telomerase. The hTERT-transduced cells were selected by incubation with puromycin (1 μg/ml), pooled, and subsequently maintained in K-SFM. All cells were incubated at 37°C in a humidified 5% CO₂ atmosphere.

Plasmids
Plasmids pHA-HIF-1α P402A/P564A-pcDNA3 and pHA-HIF-2α P405A/P531A-pcDNA3 express oxygen-insensitive variants of HIF-1α and HIF-2α, respectively [71]; they were obtained from William Kaolin via Addgene (#18955 and #18956, respectively). A HIF-1β expression plasmid, pSV-Sport-ARNT [72], was obtained from Christopher Bradfield. Plasmid pZpWT-luc contains the nt -221 to +30 region of Zp relative to the transcription initiation site cloned between the KpnI and HindIII sites of the luciferase reporter plasmid, pGL3-Basic (Promega) [73]. The mutant variants of it shown in Fig 5B contain the indicated base pair substitution mutations; they were generated by Quick Change methodology (Stratagene), with pZpWT-luc serving as template and synthetic oligonucleotides containing the desired mutations surrounded by 10 bases of wild-type sequence serving as primers. Plasmid pWTRP-luc contains the nt -1069 to +38 region of Rp relative to the transcriptional initiation site cloned between the KpnI and HindIII sites of pGL3-Basic. Plasmid pTATA-luc [74] served as a negative control. Plasmids pSG5-BZLF1 and pRTS15 (kindly provided by Diane Hayward) express Zta and Rta, respectively, from the SV40 early promoter [75]. Plasmid pcDNA3-BRLF1 expresses Rta from the CMV IE promoter [76]. Plasmid p2089 (a generous gift from Wolfgang Hammerschmidt) is a BAC containing the entire genome of the B95.8 strain of EBV [36]. 293T cells infected with the M81 strain of EBV in a BAC were a generous gift from Henri-Jacques Delecouse [77].

Chemical mimics of hypoxia
To mimic hypoxia, cells were incubated with the indicated concentrations of CoCl₂, Deferoxamine (DFO, Sigma; also called Desferoxamine, Desferal; stock solution prepared in PBS), L-Mimosine (Mim; Sigma; stock solution prepared in 10% NaHCO₃), or MLN4924 (Pevonedistat; AdooQ Bioscience #A11260; stock solution prepared in DMSO) for the indicated time periods.

Immunoblot analysis
Whole cell extracts (WCE) were prepared in SUMO lysis buffer [150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris (pH 8.0), 50 mM sodium fluoride, 50 mM β-glycerophosphate, 2 mM sodium vanadate, 1x Complete Protease Inhibitor (Roche)]. Proteins were separated by electrophoresis in SDS gels containing 4–20% (NuSep) or 10% (Biorad) polyacrylamide and transferred to nitrocellulose membranes (ISC Biosystem). After blocking by incubation for 1 h with 5% casein in TBST [10 mM Tris-HCl (pH 7.4), 0.15M NaCl, 0.1% Tween 20], the membranes were incubated overnight at 4°C in 5% casein-TBST containing antibody specific to Zta (BZLF1, 1:250, #sc-53904; Santa Cruz), Rta (BRLF1, #sc-18031, Santa Cruz), HIF-1α and HIF-2α, respectively [72]; they were maintained likewise. AGS-Akata cells, an EBV-infected clonal derivative of AGS cells (derived from a human gastric carcinoma; obtained from ATCC) [69], were maintained in F12 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) and pen-strep additionally supplemented with 400 μg/ml of G418.

293T (obtained from ATCC) is a human embryonic kidney (HEK) cell line expressing the early genes from SV40 and adenovirus. These cells were maintained in DMEM (Life Technologies) supplemented with 10% FBS and pen-strep. 293T cells harboring the B98.5 strain of EBV in BAC p2089 [36] or HRE mutant variants thereof were maintained in DMEM additionally supplemented with 100 μg/ml hygromycin B.
Hypoxia-inducible factor-1α plays roles in Epstein-Barr virus’s natural life cycle and tumorigenesis by inducing lytic infection through direct binding with Zta (Zta co-stain) or -20°C methanol (for EBNA2, Zta, and CD31 co-stains), and blocked in PBS with 0.1% Tween-20 and 5% goat
serum (EBNA2 co-stain with CD31), 5% casein, 5% goat serum (Zta co-stain with Hypoxyprobe), or 5% casein, 5% goat serum (Zta co-stain with CD31). Sections were then incubated in the indicated primary antibody overnight. The antibodies used were as follows: anti-Zta primary (1:100, BZ1; Santa Cruz) or anti-EBNA2 primary (1:50, #ab9543; Abcam), followed by goat anti-mouse IgG with Alexa Fluor 594 (1:50, #A21207; Invitrogen); and Hypoxyprobe primary (1:50, #PAb2627AP; Hypoxyprobe, Inc.) followed by donkey anti-rabbit with Alexa Fluor 594 (1:500, #A11012; Invitrogen). Images were taken and distance measurements were determined with a Zeiss Axiosmager M2 microscope and Axiosvision Software version 4.8.2.

For the IHC studies (S1 and S5 Figs), the cells and M81-induced lymphomas were fixed immediately after harvest, embedded in paraffin, sectioned, deparaffinized, the antigens retrieved by incubation with 10 mM citrate buffer (pH 6.0) containing 0.05% Tween 20 for 20 min at 98°C, and processed as previously described [46, 78, 79]. Sections were probed for the indicated proteins using the following antibodies: CD20 (1:600, clone H1; BD Biosciences); EBNA2 (1:100, PE2; Leica Microsystems); and Zta (1:200, BZ1; Santa Cruz).

For reporter assays, 293T cells maintained in 24-well plates were co-transfected using TransIT-LT1 (Mirus Corp.) with (i) 45 ng pha-HIF-1α P402A/P564A-pcDNA3 plus 45 ng phIF-1β or 45 ng of each of their parental expression plasmids as controls, and (ii) 200 ng of the indicated luciferase reporter plasmid. Cells were harvested 24- to 48-h later, lysed with Passive Lysis Buffer (Promega), and luciferase activity was determined according to the manufacturer’s instructions. All assays were performed in triplicate on three or more occasions. For all other assays, expression plasmids were transfected into the indicated cells using TransfIT-L1 and the amounts of DNA indicated following by incubation at 37°C for the times indicated prior to harvesting and processing as indicated in each figure legend.

For reporter assays, 293T cells maintained in 24-well plates were co-transfected using TransIT-LT1 (Mirus Corp.) with (i) 45 ng pha-HIF-1α P402A/P564A-pcDNA3 plus 45 ng phIF-1β or 45 ng of each of their parental expression plasmids as controls, and (ii) 200 ng of the indicated luciferase reporter plasmid. Cells were harvested 24- to 48-h later, lysed with Passive Lysis Buffer (Promega), and luciferase activity was determined according to the manufacturer’s instructions. All assays were performed in triplicate on three or more occasions. For all other assays, expression plasmids were transfected into the indicated cells using TransfIT-L1 and the amounts of DNA indicated following by incubation at 37°C for the times indicated prior to harvesting and processing as indicated in each figure legend.

Knockdown of HIF-1α

AGS-Akata cells maintained in 10-cm dishes were transiently transfected when approximately 60% confluent using TransfIT-LT1 with 0.8 μg each of five pLKO.1-based lentiviral vector DNAs encoding shRNAs that target HIF-1α (plasmids #3808, #3809, #3810, #3811, and #10819, Thermo Scientific). As controls, cells were transfected with 4 μg of pLKO.1 expressing the non-targeting shRNA 1864 (cntl. #1, #1864; Addgene) or NT (cntl. #2, #SHC002, Sigma-Aldrich). Two days later, cells were incubated with 200 μM DFO for 24 h, harvested, lysed in SUMO buffer, and processed for immunoblot analysis.

To transduce Sal cells with these shRNA-encoding lentiviruses, the lentiviruses were first packaged into virions as described by Open Biosystems. 293T cells in 10-cm-diameter dishes were co-transfected with (i) 0.8 μg of the five individual shRNA lentiviral vectors targeting HIF-1α or 4 μg of non-targeting shRNA cntl. #1 lentiviral vector, (ii) 1.4 μg of pCMV-dR8.2 dvpr (#8455; Addgene), and (iii) 0.6 μg of a plasmid encoding vesicular stomatitis virus G protein (VSV-G) (gift from Bill Sugden). The medium containing the virus was harvested 72 h later, passed through 0.8-μm-pore-size filters, and used to infect the Sal cells subsequently processed as described above for AGS-Akata cells except that the DFO was added three days after infection with the lentiviruses.

Electrophoretic-mobility-shift assays

The protein source was nuclear extract prepared as previously described [40] from AGS cells that had been incubated with 200 μM CoCl₂ for 24 h. The probe was the 5′-end-labeled, double-stranded oligonucleotide, 5′-AAACGCAAGCAGGCTTACAGATCC-3′ (underlined sequence indicates consensus HRE). Reactions were performed with 20 μM HEPES (pH 7.9), 0.1 M KCl, 6 mM MgCl₂, 4 μg poly(dl-dC)-(dl-dC), 0.5 mM PMSF, 0.5 mM DTT, 8% Ficoll in a final volume of 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl₂, and 0.1% Nonidet P-40, in the presence of 0.5 μg of HIF-1α probe. Electrophoretic-mobility-shift assays were performed as described [1].
of 20 μl. For immunoshift EMSAs, 10–100 μg of protein extract was pre-incubated in the reaction buffer for 20 min at 4°C with 1 μg anti-HIF-1α polyclonal antibody (Fab103063, Abcam) prior to addition of the radiolabeled probe and incubation at room temperature for 15 min. For competition EMSAs, unlabeled, competitor double-stranded oligonucleotides were pre-incubated with the reaction mixture prior to addition of the radiolabeled probe. Protein-DNA complexes were separated by electrophoresis at 200 V for 2 h at 4°C in a 4% non-denaturing 4% polyacrylamide gel with 0.5X Tris-borate-EDTA (TBE) as the running buffer. Gels were dried and imaged on a STORM 840 phosphorimager (GE Healthcare).

Chromatin immunoprecipitation assays

Chip assays were performed essentially as previously described [40] using approximately 2 × 10^7 SNU-719 and Sal cells grown in 15-cm dishes. Cells were incubated for 24 h with 200 μM DFO (+) or PBS (-) in medium as indicated. Protein-DNA complexes were cross-linked by incubation with 1% formaldehyde for 10 min at room temperature. Cross-linking was quenched by addition of glycine to 0.125 M. Cells were harvested by centrifugation and snap frozen until lysed. Following lysis, nuclei were isolated by centrifugation, and chromatin was sheared by sonication to approximately 500-bp size. After centrifugation to pellet debris, chromatin was divided into aliquots incubated overnight at 4°C with 2 μg of mouse anti-HIF-1α (#Ab10366; Abcam) or anti-IgG (#sc-2025; Santa Cruz) antibody as a negative control. Antibody-conjugated protein-DNA complexes were precipitated by addition of protein A-Sepharose beads (Santa Cruz), the immunoprecipitates were eluted, and the cross-links were reversed. The resulting DNAs were purified using QiAquick PCR purification kits (Qiagen) and analyzed by qPCR using Taq universal SYBR green supermix (BioRad) and the Applied Biosystems prism real-time PCR system with the following primer pairs: BZLF1: FWD 5′-GGCTGTCTATTTTTGACACCCAGC-3′, and REV 5′-AAGGTGCAATTTGTAGTGTTGACCATTACC-3′; and 4.8-kbp upstream of Zp transcription initiation site (negative control); FWD 5′-AGAAAGGGGACACACATCG-3′, and REV 5′-AATTGGAGCCTTTTG3′.

A standard curve was generated from the threshold cycle (Ct) of the input DNA diluted to 5%, 1%, and 0.2% with distilled water containing 100 μg/ml sheared salmon sperm DNA (Ambion), with percent input bound calculated relative to this standard curve. Assays were performed in triplicate on two separate occasions.

Construction of EBV mutant genomes

The 3-bp substitution mutations, HRE mt2 and HRE mt4, were introduced into the Zp HRE element in the EBV-containing BAC p2089 [36] by two-step, phase λ Red-mediated recombination essentially as previously described [80]. In brief, the I-SceI-Kan cassette present in pEPkan-S2 was PCR-amplified using the following primer pairs: HRE mt2: FWD 5′-AGGGCTTGTAATGTCCTCATAGACACACCTAAATTAGGTGCTCCAAAACCATGCATACAGGAGTAACGGTATCAGTT-3′ and REV 5′-CCAGGGACACGCTTCTCTGATTGATCGTTGAGGACACgctAAATTTAGGTTGCTATGCGTATACCAACACATTACC-3'; HRE mt4: FWD 5′-AGGGCTTGTAATGTCCTCATAGACACACCTAAATTAGGTGCTCCAAAACCATGCATACAGGAGTAACGGTATCAGTT-3′ and REV 5′-CCAGGGACACGCTTCTCTGATTGATCGTTGAGGACACgctAAATTTAGGTTGCTATGCGTATACCAACACATTACC-3′.

The Zp sequence in these primers is underlined, with the base substitutions indicated in bold italicized small letters. These PCR products were electroporated into E. coli strain GS1783 into which BAC p2089 had been previously introduced, and inserted into p2089 by homologous recombination. Induction of the I-SceI activity encoded by GS1783 led to cleavage at the unique SceI site within the BAC. Intra-molecular recombination between two copies of Zp resulted in precise removal of the inserted Epkan-2 sequences, leaving behind one copy of Zp. Clones containing the desired HRE mutant BACs were initially identified by PCR screening and, subsequently, by DNA sequence analysis of the Zp and Zta-coding regions of the BAC. The mutant variants of p2089 were then thoroughly checked for absence of large deletions, insertions and rearrangements by analysis of multiple restriction enzyme fragment patterns as previously described [43, 81] and for extraneous base-pair substitution mutations by high throughput sequence analysis as described below after recovery of the DNAs from mutant-infected 293T cell lines.

Isolation of WT- and HRE mutant-infected 293T cell lines

293T cells were transfected with twice CsCl2-purified BAC DNA and selected for hygromycin-resistance as previously described [43]. By 3-to-4 weeks post-transfection, all of the colonies of cells were GFP-positive. These clones were picked, grown up, and stored in liquid nitrogen. Their ability to produce infectious virus was determined as previously described [35] following transfection with plasmids that express the EBV Zta and gp110 proteins. The titers of the mutant virus stocks ranged from 10^4 to 10^5 green Raji units (GRU)/ml.

Sequence analysis of EBV HRE mutants

We recovered the BAC DNAs from the HRE mutant-infected 293T cell lines by Hirt extraction as previously described [43] and introduced them into E. coli strain GS500 by electroporation. Two independent colonies obtained from each of the two mutant BACs were grown, and the BAC DNAs were isolated by alkaline lysis as previously described [43]. After purification through two cycles of centrifugation in CsCl2, the highly purified BAC DNAs were sequenced using an Ion Torrent PGM (Life Technologies). We aligned the sequencing reads to the B95.8 reference strain of EBV (V01555) with Bowtie2 [82] using default alignment parameters and removing non-aligned reads. The resulting alignments were sorted using Samtools [83]. The Genome Analysis Toolkit (GATK) Unified Genotyper ([https://www.broadinstitute.org/gatk/guide/article?id=6201]) was used to detect genetic variations compared to the EBV reference. Since regions of repetitive DNA produce incorrect alignments [87] which can manifest in downstream analyses as apparent mutations, we further investigated called mutations which occurred in the repetitive regions of the EBV genome (TRs, FRs, IR2, and IR3). A program termed EasyVariant was written and used to parse each alignment and its CIGAR string [83] that allowed both position-specific coverage depth to be calculated and percentage of each of the four nucleotides to be called at each position. Any position in which 50% or more reads indicated a mutation was treated as valid unless it occurred in a repeat region where it was likely due to an incorrect alignment. We achieved sequence coverage depth of 15- or more reads over 93% and 97% of the unique junctions of the genome for HRE mt2 and mt4, respectively. The expected mutations in the HRE (mt2 and mt4) were called as such in 100% of sequence reads, and the consensus base calls within the unique regions of the genome matched the reference genome. We also performed conventional Sanger sequencing at four locations where some reads (but still less than 50%) indicated a possible frameshift mutation; in each of these cases, no mutation was found.

http://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1006404
Hypoxia-inducible factor-1α plays roles in Epstein-Barr virus’s natural life cycle and tumorigenesis by inducing lytic infection through direct binding with the lytic immediate-early gene Zta. These roles are demonstrated using human gastric cancer xenografts generated from the cell line AGS-Akata transfected with a doxycycline-inducible HIF-1α construct. AGS-Akata cells were grown on cover slips and incubated for 24 h in the absence or presence of 200 μM DFO, a small molecule that stabilizes HIF-1α. Whole-cell extracts were prepared from these cultures by incubation at 37°C for 1.5 h, followed by lysis in RIPA buffer. The lysates were sonicated and centrifuged to remove debris. The supernatants were stored frozen until analyzed by immunoblotting.

Analysis of RNA expression data sets

SNU-719 transcript data, taken from Strong et al. [30], were analyzed using the RSEM algorithm (strand-specific option) for quantification of human gene expression [88] to calculate the relative levels of HIF-1α, HIF-2α, and HIF-3α RNA present in these cells. We likewise analyzed for relative expression of the HIF-α RNAs present in primary, endemic, EBV+ Burkitt lymphomas. These latter reads, generated through the NIH’s The Cancer Genome Atlas (TCGA) project, were obtained from the NCBI Sequence Read Archive (SRA035410, now available through the NCI Genomic Data Commons). The relative levels of the HIF-α RNAs present in primary, endemic, EBV+ Burkitt lymphomas were calculated from the data provided in Table S10 of Abate et al. [31]. The RNA expression levels of the genes shown in Fig 9A that had been generated from six cell types ranging from naïve B cells to plasma cells (38 samples total) were retrieved from the previously reported microarray datasets [89–92]. These data were normalized using the GCRMA algorithm and visualized using GenomicScape (http://www.genomicscape.com/microarray/browsedata.php?acc=GS-DT-2) [93].

Supporting information

S1 Fig. IFS and IHC for Zta to determine efficiency of EBV reactivation by DFO in AGS-Akata and Sal cells.

(A) AGS-Akata cells grown on cover slips were incubated for 24 h in the absence or presence of 200 μM DFO prior to fixing and processing for co-detection of Zta protein by IFS (green) and nuclei by staining with DAPI (blue). (B) AGS-Akata cells untreated or treated (+) with 200 μM DFO prior to harvesting, fixing, embedding in paraffin, sectioning, mounting on slides, and processing for co-detection of Zta by IHC (brown) and nuclei by counterstaining with hematoxylin (H; purple). (C) and (D), Sal cells were incubated with DFO and processed the same way as were the AGS-Akata cells in panels A and B, respectively. Brown arrows indicate locations of a few of the Zta+ cells.

S2 Fig. HRE element is not a repressor-binding site and functions independently of the ZIIR element of Zp.

(A) Basal activity levels observed with the Zp mutants in the reporter assays shown in Fig 5C. (B) Schematic showing sequence of the 6-bp HRE/ZIIR mutant analyzed in panel C. (C) Reporter assay showing failure of HIF-1αβ to activate transcription from a Zp mutant altered in the two 3’-most bases of the HRE along with the ZIIR element. Assays were performed as described in Fig 5C.

S3 Fig. Adjacent serial sections of an M81-induced lymphoma stained for the indicated items.

Protocol was the same as described in the legend to Fig 11. (A) Section co-stained for Zta (green) and Hypoxyprobe (red). (B) Section co-stained for Zta (green) and CD31 (red). (C) Section co-stained for EBNA2 (green) and CD31 (red). (D) Section stained with hematoxylin and eosin. Panels A-C were counterstained with DAPI (blue).

S4 Fig. Most Zta-positive cells present in SNU-719 xenografts grown in NSG mice also reside distal to blood vessels.

The flanks of NSG mice were inoculated with 1 x 10^7 SNU-719 cells. Thirty-three days later, the mice were injected with Hypoxyprobe and sacrificed 1.5 h later. The tumors were flash frozen, sectioned, and stored at -80°C until processed by IFS as described in the legend to Fig 11. (A,B) Shown here are two representative sections co-stained for Zta (green) and CD31 (an
endothelial marker indicative of blood vessels; red) and counterstained with DAPI (blue). Sections were photographed at the same magnification (40x).
https://doi.org/10.1371/journal.ppat.1006404.s004 (TIF)

S5 Fig. Most Zta-positive cells present in B-cell lymphomas induced by EBV in humanized mice reside distal to blood vessels.

NSG mice were inoculated i.p. with human cord blood that had been infected with the M81 strain of EBV. Thirty-three days later, the mice were sacrificed, and the tumors were processed by IHC for the indicated proteins. (A,B) Shown here are two representative sets of adjacent tumor sections stained for CD20, EBNA2, and Zta (brown) and with hematoxylin and eosin. These data are representative of data observed in over two dozen EBV+ tumors obtained in several experiments performed with cord blood from different donors. Purple and dark brown arrows point to locations of blood vessels and some of the Zta+ cells, respectively. Sections were photographed at the same magnification (40x).
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Author Contributions

Conceptualization: RJK XY BAC SCK JEM.
Data curation: KGM ZL ECJ.
Formal analysis: Ti KGM ZL ECJ.
Funding acquisition: PFL ECJ SCK JEM.
Investigation: RJK XY BAC SS DMN SM JCRM KRM DLL.
Methodology: KGM ECJ.
Project administration: PFL ECJ SCK JEM.
Resources: KRM DLL.
Software: KGM ECJ.
Supervision: PFL ECJ SCK JEM.
Writing – original draft: RJK JEM.
Writing – review & editing: RJK BAC PFL ECJ SCK JEM.

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