Interferon regulatory factor 8 regulates caspase-1 expression to facilitate Epstein-Barr virus reactivation in response to B cell receptor stimulation and chemical induction

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Abstract

Interferon regulatory factor 8 (IRF8), also known as interferon consensus sequence-binding protein (ICSBP), is a transcription factor of the IRF family. IRF8 plays a key role in normal B cell differentiation, a cellular process that is intrinsically associated with Epstein-Barr virus (EBV) reactivation. However, whether IRF8 regulates EBV lytic replication remains unknown. In this study, we utilized a CRISPR/Cas9 genomic editing approach to deplete IRF8 and found that IRF8 depletion dramatically inhibits the reactivation of EBV upon lytic induction. We demonstrated that IRF8 depletion suppresses the expression of a group of genes involved in apoptosis and thus inhibits apoptosis induction upon lytic induction by B cell receptor (BCR) stimulation or chemical induction. The protein levels of caspase-1, caspase-3 and caspase-8 all dramatically decreased in IRF8-depleted cells, which led to reduced caspase activation and the stabilization of KAP1, PAX5 and DNMT3A upon BCR stimulation. Interestingly, caspase inhibition blocked the degradation of KAP1, PAX5 and DNMT3A, suppressed EBV lytic gene expression and viral DNA replication upon lytic induction, suggesting that the reduced caspase expression in IRF8-depleted cells contributes to the suppression of EBV lytic replication. We further demonstrated that IRF8 directly regulates CASP1 (caspase-1) gene expression through targeting its gene promoter and knock-down of caspase-1 abrogates EBV reactivation upon lytic induction, partially through the stabilization of KAP1. Together our study suggested that, by modulating the activation of caspases and the subsequent cleavage of KAP1 upon lytic induction, IRF8 plays a critical role in EBV lytic reactivation.
Introduction

Epstein-Barr virus (EBV), a ubiquitous human gammaherpesvirus, is associated with malignant diseases, including Burkitt’s lymphoma, Hodgkin’s lymphoma, nasopharyngeal carcinoma, and NK/T cell lymphoma [1]. The genome of EBV is approximately 170 kb in length and encodes more than 80 genes. EBV infects both B lymphocytes and some epithelial cells and the life cycle of EBV is divided into latent or lytic phases. In the lytic phase, EBV expresses all lytic genes and progeny virus particles are packaged and released from the cell [2]. The reactivation of EBV from latent to lytic phase can be triggered by expression of two viral immediate-early gene products, ZTA (also called BZLF1 or Z) and RTA (also known as BRLF1 or R). A series of cellular factors have been shown to regulate ZTA and RTA gene expression and to affect ZTA/RTA transcriptional activity [3,4,5,6,7,8,9,10,11,12,13,14,15,16]. B cell receptor (BCR) activation is a physiologically relevant stimulus for triggering EBV reactivation from latency since this occurs not only in tumor cell lines but also in freshly isolated B cells from patients [17,18].

The interferon regulatory factor (IRF) family members (IRF1-9) are transcription factors for interferon (IFN) and IFN-inducible genes [19,20]. Members of the IRF family also play a vital role in regulation of immunity and oncogenesis [21]. Previous studies showed that several IRFs are implicated in the life cycles of herpesviruses, including EBV. For examples, IRF1, IRF2, IRF4, IRF5 and IRF7 are involved in EBV latency and virus-mediated cell transformation [22,23,24,25,26]. IRF4 synergizes with RTA encoded by murine γ-herpesvirus-68 to facilitate viral M1 gene expression [27]. IRF3 and IRF7-mediated antiviral responses are counteracted by EBV encoded proteins [28,29,30].

IRF8, also known as IFN consensus sequence-binding protein (ICSBP), is a unique transcription factor of the IRF family because it is expressed predominately in hematopoietic cells [31]. Similar to other IRFs, IRF8 contains a DNA binding domain (DBD) and interacts with other proteins (such as PU.1, IRF1, IRF2 or IRF4) through the IRF association domain (IAD). In addition, IRF8 can be tyrosine phosphorylated [32,33,34,35], SUMOylated [36] and ubiquitinated [37,38]. The DBD, IAD and post-translational modifications of IRF8 all contribute to its transcription-regulatory activities [36,39,40,41]. Phosphorylation and dephosphorylation can alter the function of IRF8 in innate immune responses and leukemia pathogenesis [34,42]. SUMO conjugation-deconjugation switches IRF8’s function as a repressor or a activator [36]. IRF8 is ubiquitinated by an E3 ligase TRIM21, which alters IRF8’s ability in IL12p40 transcription [30,37]. Knockdown of IRF8 inhibits the growth of diffuse large B-cell lymphoma [43]. IRF8 is required for apoptotic induction in myeloid cells [44]. Recently, an important study established a role for IRF4 and IRF8 in EBV-mediated B-cell transformation [45]. EBV
EBNA3C, which is expressed in cells of type III latency, interacts with and stabilizes IRF4. EBNA3C coordinates with IRF4 to downregulate IRF8, which is critical for apoptosis inhibition and thus the survival of EBV-transformed cells [45]. However, in EBV-positive B cells of type I latency, EBNA3C is not expressed and IRF4 protein level is very low while IRF8 is highly expressed [46]. Despite the high expression of IRF8 in B cells of type I EBV latency, the contribution of IRF8 to EBV lytic replication remains unknown.

Driven by these facts, we explored the role of IRF8 in the EBV lytic cycle. We demonstrated that IRF8 positively regulates EBV lytic replication through regulating caspases expression and hence caspase activation upon lytic induction and caspase activation facilitates the degradation of cellular factors that limit EBV lytic replication.

## Results

### IRF8 depletion suppresses EBV lytic replication

The previous research on IRF8 and EBV latency [45] and the high expression of IRF8 in EBV-positive B cells of type I latency prompted us to test whether and how IRF8 regulates EBV lytic replication. Here we first utilized an Akata (EBV⁺) cell line, a Burkitt’s lymphoma cell line of type I latency, as a model system to investigate the role of IRF8 in the EBV lytic cycle. Because Akata (EBV⁺) cells express surface immunoglobulin receptors of the G (κ) class (IgG) and anti-IgG cross-linking mediated BCR activation can serve as a physiologically relevant stimulus for EBV lytic reactivation [18], these cells are well-suited for investigating the contribution of cellular factors in EBV lytic replication [13,47].

To demonstrate whether IRF8 regulates EBV lytic replication, we utilized CRISPR/Cas9 technology to knockdown endogenous IRF8 in Akata (EBV⁺) B cells. We designed two sgRNAs and used a lenti-viral system to establish two IRF8-depleted pool cell lines (Fig 1A). To ensure the reproducibility of our results, at least three independent lentiviral infections were performed. The infection efficiency was approximately 20% and the experiments were performed after one to two weeks selection with puromycin when all living cells were puromycin-resistant. Compared with non-targeting control (NC), the sgRNA sg1 partially knocked down the protein expression of IRF8, while sg2 efficiently depleted IRF8 (Fig 1B). To further confirm the correct targeting of IRF8 by CRISPR/Cas9, we sequenced the genomic DNA spanning the CRISPR/Cas9 targeting region of the IRF8-sg1 and IRF8-sg2 cell lines and we found that 10 out of 22 clones for sg1 and 9 out of 14 clones for sg2 contain frame shifts (S1 Fig). To evaluate the effects of IRF8 depletion on EBV lytic replication, we triggered EBV lytic replication by anti-IgG mediated BCR cross-linking. We found that the accumulation of the EBV lytic proteins ZTA and BGLF4 was suppressed in the two IRF8-depleted cell lines upon lytic induction and that the higher IRF8 knockdown efficiency correlated with lower ZTA and BGLF4 expression (Fig 1C and 1D).

We then examined the level of lytic RNA transcripts in these cell lines. As expected, knockdown of IRF8 dramatically suppressed the expression of immediate early (ZTA and RTA) and late (BGLF2) genes (Fig 1E). To test whether IRF8 plays a role in EBV replication, we measured both intracellular and extracellular EBV genome copies following lytic induction. We found that both intracellular (Fig 1F) and extracellular (Fig 1G) viral DNA copies were significantly reduced upon IRF8 depletion. These results suggested that IRF8 acts as a key positive regulator during EBV lytic reactivation.

To further demonstrate that the observed phenotype was not due to off-target effects, we reconstituted IRF8 back into the IRF8-depleted (sg2) cells. We found that IRF8 restoration facilitated EBV ZTA and RTA protein expression compared with IRF8-depleted cells upon IgG cross-linking (S2A Fig, lanes 2–3 vs 5–6). Moreover, EBV DNA replication was also
dramatically enhanced upon IRF8 reconstitution (S2B Fig, lanes 2–3 vs 5–6). Together these results suggest that IRF8 promotes EBV replication upon lytic induction.

**IRF8-dependent caspase activation is required for EBV reactivation**

As a transcription factor, IRF8 may also regulate EBV replication through altering cellular processes. To provide insight into IRF8-regulated cellular events, we performed RNA-Seq analysis for the control and IRF8-depleted cells generated from three different lentiviral transductions. Totally we identified 253 differentially expressed genes (S1 Table). Among these genes, 196 genes were down-regulated and 57 genes were up-regulated upon IRF8 depletion (Fig 2A). Gene Ontology (GO) analysis plus manual curation of these differentially regulated genes revealed that 19 genes involved in “positive regulation of apoptosis” were significantly enriched. Interestingly, all of these genes involved in apoptosis were down-regulated in
IRF8-depleted cells (Fig 2A, red dots and Fig 2B). To validate our RNA-seq results, we selected 8 genes and analyzed their expression by RT-qPCR for both IRF8-sg1 and IRF8-sg2 cells. The down-regulation was confirmed for all those genes tested, including caspase-1 (CASP1) (Figs 2B and S3A). Consistent with the reduced mRNA expression, caspase-1 protein level was reduced in IRF8-depleted cells (Figs 2C and S3B, lane 1 vs 4). The down-regulation of apoptosis related genes suggested that IRF8 depletion may suppress apoptosis induction during EBV lytic replication upon BCR activation. To test this possibility, we monitored the cleavage of PARP and global caspase substrates containing a cleavage motif [DE(T/S/A)D]. We found that IRF8 depletion suppressed protein cleavage upon BCR activation (Figs 2C and S3B, lanes 2–3 vs 5–6).

IRF8 has been shown to positively regulate the apoptosis of myeloid cells and nonhematopoietic tumor cells [44,48,49,50]. The dramatic down-regulation of caspase-mediated protein cleavage upon IRF8 depletion suggested that IRF8 may regulate the activation of caspases. To test this possibility, we monitored the level of individual caspases and their cleaved products. Strikingly, we found that the IRF8 depletion markedly reduced the levels of caspase-3 and caspase-8 and consequently the generation of active cleaved products was also suppressed upon BCR activation. In contrast, the protein levels of caspase-2, caspase-7 and caspase-9 and their cleavage were less affected by IRF8 depletion (Figs 3A and S3C). In addition, the level of Bcl-2, an anti-apoptosis protein, increased in IRF8-depleted cells (Figs 3A and S3C, Bcl-2 blot, lanes 1–3 vs 4–6), which further contributed to IRF8-dependent inhibition of apoptosis. Except for caspase-1, the gene expression levels of other caspases, including caspase-3 and caspase-8, were not regulated by IRF8 depletion according to our RNA-seq analysis (S4 Fig and S1 Table), suggesting that IRF8 may control caspase-3 and caspase-8 protein levels through modulation of translation or protein stability rather than transcription.

Because caspase activation upon apoptotic induction can facilitate EBV lytic reactivation in other EBV-positive cell lines [51,52], we reasoned that IRF8 facilitates EBV reactivation in the
Akata (EBV⁺) cells through caspase activation. To test this hypothesis, we pretreated the Akata (EBV⁺) cells with a pan-caspase inhibitor Z-VAD-FMK and then induced EBV lytic reactivation by anti-IgG cross-linking of the BCR. Caspase inhibition strongly suppressed the expression of immediate-early (ZTA and RTA), early (BGLF4) and late (BGLF2) gene expression (Fig 3B). Consistently, the EBV ZTA and BGLF4 protein expression and viral DNA replication were also blocked by caspase inhibition (Fig 3C).

The switch from EBV latency to lytic reactivation is negatively regulated by a number of cellular factors [53]. Because caspase activation can lead to the cleavage of many cellular proteins [54,55,56], we hypothesized that those factors normally suppressing EBV lytic replication are destabilized by caspase activation upon BCR stimulation. To test this hypothesis, we monitored the levels of several proteins, including KAP1 [12,57,58,59], PAX5 [8,60,61,62], DNMT3A [63] and STAT3 [64,65,66,67,68], whose functions have been shown to maintain herpesviruses latency and suppress lytic replication/reactivation. We found that the protein levels of KAP1, PAX5 and DNMT3A, but not that of STAT3, were dramatically reduced upon lytic induction (Figs 3D and S3C, lanes 1–3) while IRF8 depletion suppressed the down-regulation of KAP1, PAX5 and DNMT3A (Figs 3D and S3C, lanes 4–6). To further test whether...
caspase activation plays a role in the de-stabilization of KAP1, PAX5 and DNMT3A, we monitored their protein levels in Akata (EBV+) cells when caspases are inhibited and lytic replication is triggered by BCR stimulation. Interestingly, pretreatment of the cells with a pan-caspase inhibitor Z-VAD-FMK restored their expression (Fig 3E). For KAP1, in addition to the reduced protein level, we also noticed the generation of two potential cleaved fragments upon BCR activation, which is also blocked by caspase inhibition (Fig 3E, KAP1, longer exposure). Taken together, these results suggested caspase activation-mediated de-stabilization of cellular restriction factors contributes to EBV lytic replication.

To demonstrate the effect of IRF8 in additional EBV-positive cell lines, we depleted IRF8 in two additional cell lines, P3HR-1 and an EBV transformed lymphoblastoid cell line (LCL). We observed universal lower reactivation for EBV in IRF8-depleted cells treated with either gemcitabine, anti-IgM (for LCL cells) or TPA/sodium butyrate (for P3HR-1 cells) (Fig 4), reinforcing that IRF8 plays a key role in EBV reactivation.

**Fig 4. IRF8 depletion suppresses EBV reactivation in LCL and P3HR-1 cells upon lytic induction.** A and B. Control (NC) and IRF8-depleted (sg1 and sg2) LCL cells were either untreated (0 hr) or treated with 1 μg/mL gemcitabine (A) or 20 μg/mL α-IgM (B) for 48 hrs to induce lytic replication. Western blot analyses showing IRF8, ZTA, caspase-1 and cleaved-PARP level as indicated. C. Control (NC) and IRF8-depleted (sg1 and sg2) P3HR-1 cells were either untreated (0 hr) or treated with TPA (20 ng/ml)/sodium butyrate (NaBu, 3 mM) for 48 hrs to induce lytic replication. Western blot analyses showing IRF8, ZTA and caspase-1 level as indicated.

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IRF8 regulates CASP1 (caspase-1) promoter activity

Based on our RNA-seq results, only CASP1 (caspase-1) gene was regulated by IRF8 at the RNA level (S4 Fig). Previous studies using ChIP-seq showed that IRF8 could bind to the promoter regions of both human and mouse CASP1 at a conserved consensus site, -40 to -31 bp upstream of the start codon of human CASP1 (Fig 5A) [69,70]. However, it is not clear

Fig 5. IRF8 regulates CASP1 promoter activities. A. Schematic representation of the promoter of human CASP1. IRF8 consensus binding site is highlighted in green. The ATG of CASP1 is highlighted in red. The pGL2-CASP1p constructs (with or without IRF8 consensus site) and the IRF8 consensus site mutated construct were co-transfected into 293T cells with either vector control or IRF8 expression vectors. Luciferase assays were performed 36 hrs post-transfection. The value of cells transfected with empty vectors was set as 1. The results were presented as mean ± standard deviation of triplicate assays. ** p<0.001.

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whether IRF8 directly regulates \textit{CASP1} expression. We hypothesized that IRF8, as a transcription activator, directly regulates \textit{CASP1} gene expression through binding to its promoter. To test our hypothesis, we constructed luciferase reporter plasmids, which contain the \textit{CASP1} promoter with or without the putative IRF8 binding site (Fig 5B). The luciferase reporter assay showed that IRF8 activated the wild-type \textit{CASP1} promoter but not the truncated version without the IRF8 binding site (Fig 5B). To confirm our results, we mutated the conserved IRF8 binding site and found that IRF8 failed to activate the mutated reporter (Fig 5B). To further validate our results, we constructed a DNA-binding deficient IRF8 mutant (K108E) [71] and tested whether it can block the activation of \textit{CASP1} promoter. Compared with wild-type IRF8, the DNA-binding deficient mutant (K108E) lost the ability to regulate the \textit{CASP1} promoter (Fig 5C). In conclusion, our results demonstrated that IRF8 enhances \textit{CASP1} gene expression through regulation of its promoter. A previous study showed that IRF1 can also regulate \textit{CASP1} gene promoter [72]. Therefore, we tested whether IRF1 could cooperate with IRF8 to further enhance the \textit{CASP1} promoter activity. The luciferase reporter assay demonstrated that IRF1 synergized with IRF8 to further enhance \textit{CASP1} promoter activity (Fig 5D).

To further prove whether IRF8/IRF1 bind to \textit{CASP1} promoter in B cells, we performed ChIP experiments using chromatin prepared from EBV-positive Akata, LCL and P3HR-1 cells. Our results showed that IRF8/IRF1 indeed bind to the promoter region of \textit{CASP1} for all these cells (S5A Fig), suggesting that they directly regulate \textit{CASP1} expression \textit{in vivo}. To demonstrate physiological relevance of IRF8/IRF1 activation of \textit{CASP1} promoter observed in 293T cells, we performed luciferase assay using Akata cells. Similarly, we found that IRF8 and IRF1 triggered a strong activation of \textit{CASP1} promoter while the IRF8 DNA binding deficient mutant (K108E) failed to activate the promoter (S5B Fig).

Our RNA-seq analysis showed that both \textit{IRF1} and \textit{IRF8} are expressed in the Akata (EBV\textsuperscript{+}) cells, with \textit{IRF8} level approximately 6-fold higher than that of \textit{IRF1} (S6 Fig). Based on the luciferase assay, IRF8, together with its closely related family member IRF1, plays an effective role on regulating \textit{CASP1} expression.

\textbf{Caspase-1 depletion abrogates EBV lytic replication}

The control of \textit{caspase-1} expression by IRF8 promoted us to test whether caspase-1 contributes to EBV reactivation upon lytic induction. To answer this question, we utilized a similar CRISPR/Cas9 approach to deplete endogenous \textit{CASP1} in Akata (EBV\textsuperscript{+}) B cells. To offset the potential off-target effect, we designed two sgRNAs to establish \textit{CASP1}-depleted cell lines by three distinct lentiviral infections (Fig 6A). To further confirm the correct targeting of \textit{CASP1} by CRISPR/Cas9, we also sequenced the genomic DNA spanning the CRISPR/Cas9 targeting region of the \textit{CASP1}-sg1 and \textit{CASP1}-sg2 cell lines. The sequencing results showed that frame shifts were introduced in 8 out of 13 clones for \textit{CASP1}-sg1 and 12 out of 14 clones for \textit{CASP1}-sg2 (S7 Fig). To evaluate the effects of caspase-1 depletion on EBV lytic reactivation, we triggered EBV reactivation by anti-IgG mediated BCR cross-linking. We found that the accumulation of the EBV lytic proteins ZTA and RTA was dramatically suppressed in the two \textit{CASP1}-depleted cell lines upon BCR activation (Fig 6B). We also examined the level of lytic RNA transcripts in these cell lines. As expected, knockdown of \textit{CASP1} dramatically suppressed the expression of immediate early (ZTA and RTA) and late (BGLF2) genes (Fig 6C). To test whether caspase-1 plays a role in EBV replication, we measured intracellular EBV genome copies following lytic induction. Compared with control, the intracellular viral DNA copies were significantly reduced upon \textit{caspase-1} depletion (Fig 6D), suggesting that caspase-1 is required for EBV reactivation.
To demonstrate the effect of caspase-1 in broader settings, we also depleted CASP1 in P3HR-1 and EBV transformed LCL cells. We found that CASP1-depletion suppresses EBV reactivation treated with either gemcitabine, anti-IgM (for LCL) or TPA/sodium butyrate (for P3HR-1) (Fig 7), suggesting that IRF8/caspase-1 axis contributes to EBV reactivation upon lytic induction.

Caspase-1 promotes EBV reactivation partially through KAP1 cleavage

IRF8 can affect the degradation of KAP1, PAX5 and DNMT3A through caspase activation (Figs 3D and S3C). To test whether caspase-1 could affect their degradation, we monitored the protein stability when caspase-1 was depleted and lytic reactivation was induced by BCR activation. Interestingly, we found that the degradation of KAP1, but not PAX5 and DNMT3A, was blocked in caspase-1-depleted cells (Fig 8A). Based on these results, we reasoned that KAP1 might be cleaved by caspase-1. To prove this, we performed an in vitro cleavage assay using individual recombinant caspases and KAP1. To facilitate the detection of cleaved KAP1 fragments, we utilized an N-terminally HA-tagged KAP1 construct and immunoprecipitated the KAP1 protein from transfected 293T cells using HA magnetic beads. HA-KAP1 was eluted

Fig 6. CASP1 depletion inhibits the reactivation of EBV in Akata (EBV+) cells. A. The locations of two sgRNAs (sg1 and sg2) used for CASP1 depletion. B-D. CASP1-depleted (sg1 and sg2) and control (NC) Akata (EBV+) cells were either untreated (0 hr) or treated with anti-IgG for 24 and 48 hrs to induce lytic replication. The cell pellets were harvested 24 and 48 hrs after anti-IgG stimulation. Protein extracts were analyzed by western blot using antibodies against CASP1 and EBV immediate-early (ZTA and RTA) proteins and β-actin (B). RT-qPCR showing the suppression of EBV immediate-early (ZTA and RTA) and late (BGLF2) genes expression upon CASP1 depletion (sg1 and sg2) (C). qPCR showing the reduction of intracellular viral DNA copy numbers upon CASP1 depletion (D). The EBV genome copy number was measured by qPCR using primers specific to EBV BALF5. The intracellular EBV copy number was normalized by qPCR using specific primers to β-actin.

Data are presented as means ± standard deviations (n = 3). **p<0.01.

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To demonstrate the effect of caspase-1 in broader settings, we also depleted CASP1 in P3HR-1 and EBV transformed LCL cells. We found that CASP1-depletion suppresses EBV reactivation treated with either gemcitabine, anti-IgM (for LCL) or TPA/sodium butyrate (for P3HR-1) (Fig 7), suggesting that IRF8/caspase-1 axis contributes to EBV reactivation upon lytic induction.
for the in vitro cleavage assay. Anti-HA and anti-KAP1 antibodies recognize N- and C-terminal of KAP1 respectively (Fig 8B), which facilitates the detection of cleaved fragments. Interestingly, we found that caspase-1, as well as caspase-8 can cleave KAP1 in vitro (Fig 8B). We also checked the expression of caspase-8 (CASP8) and found that the caspase-8 protein level (Fig 8A) but not its mRNA level (S8 Fig) was also reduced in caspase-1-depleted cells. These results together suggested that KAP1 cleavage is regulated by caspase-1 and -8 in Akata (EBV+)) cells upon lytic induction. Because KAP1 depletion has been shown to facilitate EBV, Kaposi’s sarcoma-associated herpesvirus (KSHV) and human cytomegalovirus reactivation [12,57,58,59], we reasoned that the cleavage of KAP1 by caspase-1 and -8 should promote viral reactivation.

To prove our prediction, we further depleted KAP1 in CASPI-depleted (sg1) Akata cells by CRISPR/Cas9 genomic editing approach. As expected, KAP1-depletion in CASPI-depleted cells restored EBV reactivation upon BCR activation (Fig 9). Taken together, our results suggested that KAP1 is one of the important downstream targets of caspase-1 critical for EBV reactivation.

Discussion
In this study, we discovered that the cellular factor IRF8 facilitates EBV lytic replication by promoting caspase expression and their activation upon lytic induction. The IRF family proteins

Fig 7. CASPI depletion suppresses EBV reactivation in LCL and P3HR-1 cells upon lytic induction. A and B. Control (NC) and CASPI-depleted (sg1 and sg2) LCL cells were either untreated (0 hr) or treated with 1 μg/mL gemcitabine (A) or 20 μg/mL α-IgM (B) for 48 hrs to induce lytic replication. Western blot analyses showing caspase-1, ZTA and RTA level as indicated. C. Control (NC) and CASPI-depleted (sg1 and sg2) P3HR-1 cells were either untreated (0 hr) or treated with TPA (20 ng/ml)/sodium butyrate (NaBu, 3 mM) for 48 hrs to induce lytic replication. Western blot analyses showing caspase-1, ZTA and RTA level as indicated.

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have been shown to play an important role in immunity, cell growth, differentiation and oncogenesis [19]. In contrast to the positive role of IRF8 in EBV lytic replication observed in our study, most of the IRFs contribute to anti-viral immunity and block the infection or lytic reactivation of herpesviruses. For example, it was reported that IRF1 restricts gammaherpesvirus replication through IFN-mediated suppression of viral replication [73,74,75,76]. IRF2 also suppresses gammaherpesvirus replication and reactivation by inhibiting the M2 gene promoter [77]. Herpesviruses have evolved strategies to block IRF3 mediated anti-viral signaling [30,78]. IRF5 or IRF7-mediated suppression of KSHV replication is counteracted by virally encoded proteins [79,80,81]. While IRF4 has been implicated in suppressing KSHV replication [82,83,84], it has been shown that IRF4 promotes gammaherpesvirus-68 replication through
enhancing viral promoter activation [27,85,86]. Our identification of IRF8 as a positive regulator for EBV reactivation provides another example of IRFs in promoting herpesvirus lytic replication.

IRF8 is a unique member of the IRF family. It is highly expressed in B cells [87] and plays a critical role in B cell biology [88]. A recent study showed that IRF8 regulates EBV latency and the apoptosis of EBV-positive B cells [45]. However, the contribution of IRF8 to EBV lytic replication remained unclear prior to our study.

Using a CRISPR/Cas9 genomic editing method, we for the first time demonstrated that IRF8 depletion dramatically suppresses the reactivation of EBV (Figs 1 and 4). IRF8 positively regulates apoptosis in different types of cells, including B cells [44,48,49,50,89]. Our RNA-seq and western blot analyses showed that IRF8 modulates caspase activation during EBV lytic replication (Figs 2 and 3). Especially, IRF8 binds to and enhances CASP1 gene promoter activity (Fig 5) and caspase-1 expression is critical for EBV reactivation (Figs 6 and 7), partially through KAP1 cleavage (Figs 8 and 9). The regulation of caspase-1 by IRF8 may also contribute to subsequent BPLF1 cleavage, which has been shown to facilitate EBV DNA replication [90]. In addition, the cleavage of other cellular [56,91,92,93,94] or potentially viral proteins by caspase-1 and other caspases could also contribute to EBV reactivation. In addition to caspase cleavage of BPLF1, caspase-3 was reported to cleave LMP1 in Hela cells while the functional importance is not clear [95]. Using bioinformatic tools PeptideCutter and GraBCas [96,97], we also predicted the potential caspase cleavage sites for EBV proteins and found that many other viral proteins, such as BCRF1/vIL10, may also be potentially cleaved by caspases (S2 Table). Further detailed studies are required to prove their cleavage and the subsequent functional importance during EBV reactivation.

Several studies showed that EBV lytic reactivation is closely associated with apoptosis and that caspase activation promotes EBV lytic replication in EBV-transformed LCLs and EBV-infected gastric cancer (AGS) cells [51,52]. However, the underlying mechanisms for caspase
activation in EBV lytic replication were not clear. Here we provide evidence that caspase activation induces de-stabilization of cellular factors KAP1, PAX5 and DNMT3A contributes to efficient EBV replication (Fig 3). KAP1 is a corepressor that inhibits the reactivation of multiple herpesviruses [12,57,58,98,99]. Although phosphorylation of KAP1 overcomes KAP1-mediated inhibition, our study suggested that caspase-1/-8-mediated cleavage provides another means to antagonize KAP1-mediated inhibition (Fig 8). PAX5 is a B-cell-specific transcription factor that promotes EBV latency and suppresses lytic reactivation [8,60,61,62]. A previous study suggested that the lytic triggers TPA and sodium butyrate facilitate PAX5 destabilization through down-regulation of its mRNA expression [8] and BCR stimulation of B cells also decreases the level of PAX5 mRNA [100]. Our demonstration of caspase activation in PAX5 degradation provides an additional layer of regulation of PAX5 during EBV lytic replication. The de novo DNA methyltransferase DNMT3A contribute to γ-herpesvirus latency by suppressing viral lytic gene promoters through methylation [63]. It is conceivable that the down-regulation of DNMT3A by caspase activation would facilitate viral lytic replication.

Recent studies suggested that not only the decrease of IRF8 but also the increase of IRF4 is required for B cell differentiation, and that the IRF4/IRF8 ratios provide the differential signal for plasmablast versus germinal center plasma cell fate [70,88]. In Akata (EBV+) B cells, the expression of IRF4 is very low revealed by RNA-Seq (S6 Fig) and IRF4 protein is not detectable by western blot analysis [46]. Therefore, in the absence of IRF4, IRF8 depletion may not be sufficient to trigger B cell differentiation.

Although IRF8 normally suppresses B cell differentiation to plasma cells [101], a process that positively contributes to EBV reactivation [7], the results of our current work and the studies of others support a model in which IRF8 facilitates the reactivation of EBV upon lytic induction (Fig 10). IRF8 plays a key role in maintaining caspase-1 expression, a cellular protease critical for EBV reactivation upon lytic induction. Caspase-1 activation can trigger the specific cleavage of EBV BPLF1 for efficient viral DNA replication [90]. The activation of caspase-1 and caspase-8 can lead to the cleavage and destabilization of KAP1 and thus enhanced EBV replication.

As a positive regulator of interferon signaling, IRF8 might also function as an anti-viral factor [102,103,104] by promoting interferon signaling during primary EBV infection, which could then limit viral lytic infection and facilitate the establishment of latency. Future studies are required to examine this possibility.

In summary, our study suggests that IRF8 positively regulates EBV lytic replication upon lytic induction. These findings provide valuable insights into our understanding of IRF8 and caspase activation in EBV lytic replication, which lays the foundation for developing novel therapeutic strategies against EBV-associated malignancies.

Materials and methods
Cell culture and reagents
Akata (EBV+) cells (gifts from Diane Hayward, Johns Hopkins University) were grown in RPMI 1640 media supplemented with 10% FBS (Cat# 26140079, Thermo Fisher Scientific) in 5% CO2 at 37°C [105,106]. The P3HR-1 cell (ATCC, HTB-62) was purchased from ATCC. The EBV-transformed lymphoblast cell lines (LCL, GM11830) was purchased from the Coriell Institute for Medical Research (Camden, NJ). The P3HR-1 cell was grown in RPMI 1640 media supplemented with 10% FBS. The LCL cell was cultured in RPMI 1640 media supplemented with 15% FBS. 293T cells (a gift from Diane Hayward, Johns Hopkins University) were grown in DMEM media supplemented with 10% FBS. The pan-caspase inhibitor (Z-VAD-FMK, Cat# A1902) was purchased from ApexBio.
Plasmids, cloning, and site-directed mutagenesis

Plasmid DNA was purified on miniprep columns according to the manufacturer’s protocol (Qiagen). pCMV3-N-FLAG and pCMV3-N-FLAG-IRF8 were obtained from Sino biological. pcDNA3.1-V5-His and pSG5 were obtained from Invitrogen and Stratagene, respectively. T vector pMD19 was bought from Clontech. pSG5-HA-KAP1 expression vector (pGL190) was a gift from Diane Hayward (Johns Hopkins) and contain the corresponding open reading frames in a derivative of pSG5 (Stratagene) [107]. The IRF1 ORF was cloned from Akata (EBV+) cDNA into pMD19 (Clontech) by PCR using the following primer sets: forward (5’-ATGCCCATCACTCGGATGC-3’) and reverse (5’-CTACGGTGCAACAGGAATGG-3’). IRF1 was then subcloned into pcDNA3.1-V5-His (Invitrogen) by using Gibson assembly and the following two primer sets: primer set-1, forward (5’-CCAGTGTGGTACTCGCATCTCAG-3’) and reverse (5’-CATTTTACCAACAGCATGATGC-3’); primer set-2, forward (5’-CAGGCCTCCCTGACCCGAAGCTTGGGATTTCCGATCT-3’) and reverse (5’-CTTTCGACCCGAAGCTTGGGATTTCCGATCT-3’).
reverse (5’-GGGACATGCCAGTGATTGGCGATAGTAAGGGCAATTCCACCAACACACTG-3’). The pGL2-CASP1p1 (−488 to −8 relative to the CASP1 ORF) and pGL2-CASP1p2 (−488 to −66) luciferase reporter plasmids were constructed into the pGL2-basic vector (Promega) by using the Gibson assembly and the following two primer sets for pGL2-CASP1p1: primer set-1, forward (5’-GCTCTTACGCGTCTAGCTGAGTGTGAAAAGAAGGAAATTTAAGAA-3’) and reverse (5’-CAACAGTACCGGAATGCGCAAGCTTCTCTCCCTGTTGTAAGACAGC-3’); primer set-2, forward (5’-GTCACACACAGGAAGGAGAGGAAGAGTCTTGCGACGCTTGTAC-3’) and reverse (5’-TTCTATTTAATGTCCTTCTTTTCACACTCGAGCTAGACGACGG-3’); and the following two primer sets for pGL2-CASP1p2: primer set-1, forward (5’-GCTCTTACGCGTCTAGCTGAGTGTGAAAAGAAGGAAATTTAAGAA-3’) and reverse (5’-CAACAGTACCGGAATGCGCAAGCTTCTCTCCCTGTTGTAAGACAGC-3’); primer set-2, forward (5’-GTCAGACTATCCCTGGACGCTGTTGTTGAG-3’) and reverse (5’-TTCTATTTAATGTCCTTCTTTTCACACTCGAGCTAGCGACGACGG-3’).

Plasmids pCMV3-N-FLAG-IRF8(K108E) and pGL2-CASP1p1-mut (IRF8 binding site mutation) were constructed by using QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) and the following primer sets: IRF8(K108E) forward (5’-GGACATTTCCGAGCCATACGATTCTTGTTATCCGGAAGGACGACGG-3’) and reverse (5’-CAAGGAAAGGAATGCGCAAGCTTCTCTCCCTGTTGTAAGACAGC-3’); CASP1p1-mut forward (5’-CCAAAAAGGAAGGCGAAGCATACTTTCAGTGGAAAGAGAAGCTTG-3’) and reverse (5’-CAAGGAAAGGAATGCGCAAGCTTCTCTCCCTGTTGTAAGACAGC-3’); DNA sequences in all these plasmids were authenticated by automatic sequencing.

IRF8, CASP1 and KAP1 depletion by CRISPR/Cas9 genomic editing

To deplete IRF8 or CASP1, two different sgRNAs targeting human IRF8 or CASP1 were designed and cloned into lentiCRISPR v2 vector (a gift from Feng Zhang; Addgene plasmid #52961) [108]. Packaging 293T cells were transfected with IRF8 or CASP1 sgRNAs or negative controls (non-targeting sgRNA-NC) and helper vectors (pMD2.G and psPAX2; gifts from Didier Trono; Addgene plasmid #s 12259 and 12260) using Lipofectamine 2000 reagent (Cat# 11668019, Life Technologies). Medium containing lentiviral particles and 8 μg/mL polybrene (Sigma-Aldrich, St. Louis) was used to infect Akata (EBV+) cells. Infected cells were selected in medium containing 2 μg/mL puromycin.

To deplete KAP1, two different sgRNAs targeting human KAP1 were designed and cloned into lentiCRISPR v2-Blast vector (a gift from Mohan Babu, Addgene plasmid #83480). Packaging 293T cells were transfected with KAP1 sgRNAs or negative controls (non-targeting sgRNA-NC) and helper vectors (pMD2.G and psPAX2) using Lipofectamine 2000 reagent. Medium containing lentiviral particles and 8 μg/mL polybrene were used to infect caspase-1 knockout cell lines. Infected cells were selected in medium containing 10 μg/mL blasticidin.

The target guides sequences are as follows: IRF8-sg1: forward (5’-CACCGATTGACAGTCGATGTCCACCGTGAC-3’) and reverse (5’-AAACGGATACTGGAGCTCACCCATGAC-3’); IRF8-sg2: forward (5’-CACCGCGGATACTGGAGCTCACCCATGAC-3’) and reverse (5’-AAACGTTTTCATAGGAGCACTCAGTAAAGAA-3’); CASP1-sg1: forward (5’-CACCGCCAGATGTCCACCGTGAC-3’) and reverse (5’-AAACGGATACTGGAGCTCACCCATGAC-3’); CASP1-sg2: forward (5’-CACCGATTGACAGTCGATGTCCACCGTGAC-3’) and reverse (5’-AAACGGATACTGGAGCTCACCCATGAC-3’); sgRNA-NC: forward (5’-AACCGTGATGTCCACCGTGAC-3’) and reverse (5’-AAACCGTGATGTCCACCGTGAC-3’); KAP1-sg1: forward (5’-CACCGCGGATACTGGAGCTCACCCATGAC-3’) and reverse (5’-AAACGGATACTGGAGCTCACCCATGAC-3’);
Sequencing of CRISPR targeting region

IRF8 or CASP1 knockdown efficiency was confirmed using western blot analysis and Sanger sequencing. In details, the PAM region (containing the target site of sgRNA) was amplified from DNA mixture extracted from three biological IRF8-sg1, IRF8-sg2, CASP1-sg1 and CASP1-sg2 pool cells, respectively by using Wizard Genomic DNA Purification Kit (Fisher). The primer sets used for cloning are as follows: IRF8-sg1: forward (5'-AATGGTGGTGGTCGGCG GCTTC-3') and reverse (5'-GCCTGGGAGTTTTAAGGGGAAG-3') and reverse (5'-TCCATTAACCTTTGATGCT CCGAAA-3'); CASP1-sg1: forward (5'-TCAATTCCTTCCCCTTTCTAAT-3') and reverse (5'-AGGCTTGTGCTGCATGA CTCTTAT-3'); CASP1-sg2: forward (5'-TGGGCTATTTCTGC TCTTACATTCTT-3') and reverse (5'-CCTTTCGGAATAACGGAGTCAATC-3'). The PCR amplicons were subcloned into pMD19 vectors (Clontech) and more than 10 clones were randomly chosen for sequencing.

RNA-seq analysis

Total RNA from three biological replicates (cells derived from three distinct lentiviral transductions) was extracted using ISOLATE II RNA Mini Kit (Bioline). The library construction, cluster generation and HiSeq (Illumina) sequencing were performed with by the Genomics Sequencing Core of the Department of Environmental Health (University of Cincinnati) following the previous reported methods [109]. Raw fastq data were analyzed by using Galaxy (https://usegalaxy.org/). Human genome (hg38) was used as the reference genome. Differential gene expression between IRF8-depleted (IRF8-sg2) and control (NC) cells was analyzed by using DESeq2 [110]. The differentially expressed genes were selected based on a false-discovery rate–adjusted q-value (q < 0.05). Genes with more than 2-fold change were selected for further analysis. RNA-seq raw data have been submitted to National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA; accession numbers: SRP107862) with access URL https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP107862.

Chromatin-immunoprecipitation (ChIP)

2x10^7 Akata (EBV*), LCL and P3HR-1 cells were cross-linked individually in 1% (w/v) formaldehyde (Sigma) for 5 min at room temperature and the cross-linking reaction was quenched by addition of glycine to a final concentration of 0.125M. Cells were washed twice with cold PBS and lysed in 1 ml of cell lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM NaCl, 0.2% [v/v] NP40, 10 mM Sodium butyrate, 50 μg/ml PMSF) with fresh added complete protease inhibitor on ice for 10 min. After centrifuge at 2,500 rpm at 4˚C for 5 min, the supernatant was discarded and the nuclei were resuspended in 1.2 ml nuclei lysis buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, 1% [w/v] SDS, 10 mM Sodium butyrate, 50 μg/ml PMSF) with fresh added complete protease inhibitor on ice for 10 min. Then sonication was performed with a Diagenode Bioruptor 300. After extract clearing by centrifugation, supernatants were diluted 1:10 in dilution buffer (20 mM Tris-HCl [pH 8.1], 150 mM NaCl, 2 mM EDTA, 1% [v/v] Triton X-100, 0.01% [w/v] SDS, 10 mM Sodium butyrate 50 μg/ml PMSF) with fresh added complete protease inhibitor. Aliquots of each input chromatin lysate were reserved for PCR analysis. 1 ml of diluted chromatin lysate was incubated with ChIP-grade antibodies with rotation at 4˚C overnight. Primary antibodies used were anti-IRF8 (Santa Cruz, Cat # sc-6058X), anti-CASP1 (Abcam, Cat # ab206890), and anti-EBV lytic replication (PLOS Pathogens | https://doi.org/10.1371/journal.ppat.1006868 January 22, 2018 17 / 28
normal goat IgG (Santa Cruz, Cat # sc-2028), anti-IRF1 (abcam, Cat # ab26109), and normal rabbit IgG (Santa Cruz, Cat # sc-2027). 25 μl Protein A/G magnetic beads (life technologies, 10002D and 10004D) were added to each 1 ml ChIP and incubated for 2 hour at 4˚C with rotation. Next, magnetic beads were pelleted with magnetic separation rack and washed once with cold low salt wash buffer (20 mM Tris-HCl [pH8.1], 2 mM EDTA, 150 mM NaCl, 1% [v/v] Triton X-100, 0.1% [w/v] SDS), once with high salt wash buffer (identical to low salt wash buffer, except 500 mM NaCl), once with LiCl wash buffer (10 mM Tris-HCl [pH8.1], 1 mM EDTA, 0.25 M LiCl, 1% [v/v] NP40, 1% Deoxycholic acid), and finally twice with TE buffer (10 mM Tris-HCl [pH8.1], 1 mM EDTA). Samples were then resuspended in 150 μl of elution buffer (0.1 M NaHCO3, 1% [w/v] SDS) and rotated for 20 min at room temperature. Two rounds of elution of protein-DNA complexes were pooled. Reversal of cross-linking was accomplished by incubation of pooled eluates at 65˚C for 4 hours after addition of NaCl to final concentration of 200mM and 100 μg/ml Proteinase K. DNA was purified by phenol-chloroform extraction followed by isopropanol-sodium acetate precipitation and then resuspended in 100 μl nuclease-free water and quantified using regular PCR. Purified input chromatin lysate was used in PCR reactions for standardization. ChIP primers used to amplify the CASP1 promoter are: forward (5’-TACACTACCTGATGCAGGCTA-3’) and reverse (5’-TGAAACTGAGGAATGCTTCG-3’).

Reverse transcription and quantitative PCR (RT-qPCR)
Total RNA was extracted using ISOLATE II RNA Mini Kit (Bioline). Reverse transcription was carried out by using High Capacity cDNA Reverse Transcription Kit (Invitrogen). Quantitative PCR (qPCR) was performed using an ABI Prism 7000 Sequence Detector with SYBR Green. The PCR reactions were set up in a 96-well optical plate in duplicate by adding the following reagents into each well: 2 μl of cDNA, 10 μl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA); the final concentrations of primers were 0.3 μmol/L in a final volume of 20 μl. The PCR amplification protocol was initiated at 50˚C for 2 min followed by 10 min at 95˚C and 40 PCR cycles consisting of 15 seconds at 95˚C followed by 60˚C for 1 min. All samples were tested with the reference gene β-actin for data normalization to correct for variations in RNA quality and quantity. The specificity of amplification of targets with high Ct values was confirmed by analysis of the temperature dissociation curves. Primers used for measuring gene transcriptional level: RTA and β-actin primers were described previously [13]; ZTA primers are forward 5’-AGGCCAGCTCACCTGCACTC-3’ and reverse 5’-TGATTCTGAGTTATGTCTGA-3’; BGLF2 primers are forward 5’-ATCTGGCACCTGTCCTTTGCT-3’ and reverse5’-GGGACCTCTTTCCATTAGGC-3’; BGLF4 primers are forward 5’-GGCAATAGAGGGATAGAGG-3’ and reverse 5’-TGGTCTGCTGAGGTTATGATG-3’; CASP1 primers are forward 5’-ATACTGGGGTTGTCCTGTG-3’ and reverse 5’-GCTTAAATTTGCTTCACATA-3’; AIM2 primers are forward 5’-TAGCGCCTCACGTGTTTAGC-3’ and reverse 5’-TGAAACGCGTTGATCTTCG-3’; IFNB1 primers are forward 5’-CAGGAGAGCAATTTGGAGGA-3’ and reverse 5’-CTTGGAGGTTCTTCTCTGCT-3’; SLAMF7 primers are forward 5’-GAACCGACAGCTCTCTTACC-3’ and reverse 5’-AATATGGCTTTGTTCCACCAAC-3’; SULF1 primers are forward 5’-ATCTGGGTGAATCAATCTC-3’ and reverse 5’-ATGGCAGATTCAATTTTCG-3’; TNFSF10 primers are forward 5’-AGCAATGCCACTTTTGGAGTG-3’ and reverse 5’-TTACAGTGCTCTGCTGCT-3’; MX1 primers are forward 5’-GATCAGAGGATGTTGCC-3’ and reverse 5’-AGCTGGGAAACGACTTTC-3’; DAPL1 primers are forward 5’-TGCCCTGAAATGAGCCTGTG-3’ and reverse 5’-TGAGGATTTTTTGTGAGCCAT-3’; CASP8 primers are forward 5’-TGTCAGATTCTCCCAATAA-3’ and reverse 5’-GGTCACCTGAACTTTGGGAA-3’.
**IRF8 reconstitution**

The pLX304 vector was a gift from David Root (Addgene plasmid # 25890). The V5-tagged pLX304-IRF8 was purchased from DNASU Plasmid Repository. To prepare lentiviruses, 293T cells were transfected with empty vector or pLX304 containing the gene of IRF8 and the help vectors (pMD2.G and psPAX2) using Lipofectamine 2000 reagent. The supernatants were harvested at 48 h after transfection. The medium containing lentiviral particles and 8 μg/mL polybrene were used to infect IRF8-depleted (sg2) cell lines. Infected cells were selected in medium containing 10 μg/mL blasticidin.

**Luciferase reporter assay**

Luciferase assay was performed as previously described [16]. Briefly, 293T cells were co-transfected with the firefly luciferase reporter vectors along with IRF8 (WT or K108E mutant), IRF1, and renilla expression plasmids using Lipofectamine 2000 reagent (Cat# 11668019, Life Technologies). The Akata (EBV⁺) cell was transfected using electroporation method. For plasmid transfection, 10 μg each of plasmid were mixed with 5x10⁶ cells in a 4-mm cuvette. Electroporation was performed at 970 μF and 0.2 V with a Gene pulser Xcell system (Bio-Rad). The cells were transferred to new plates contain 10 ml pre-warmed fresh medium. At thirty-six hours post-transfection, cell extracts were prepared and assayed with the dual-luciferase assay kit from Promega (Cat #E1960, Madison, WI, USA). Each condition was performed in triplicate.

**Lytic induction and measurement of viral DNA copy number**

Akata (EBV⁺) cells were treated with 50 μg/ml of goat anti-human IgG (MP Biomedicals) for 24 and 48 h to induce the EBV lytic cycle. For caspase inhibition assay, Akata (EBV⁺) cells were untreated or pretreated with pan-caspase inhibitor for 1 hr and then treated with anti-IgG (1:200, Cat# 55087, MP Biomedicals) for additional 48 hrs. EBV reactivation in P3HR-1 cells was triggered by addition of TPA (20 ng/ml) and sodium butyrate (3 mM; Millipore, Cat# 19–137). The EBV lytic replication in LCL cells was induced by addition of gemcitabine (1 μg/ml; Fisher Scientific, Cat# NC9325685). To induce the BCR activation, the LCL cells were treated with anti-IgM antibody (20 μg/mL, Cat# 2020–01, Southern Biotech) for 0 to 48 hrs.

To measure EBV replication, intracellular viral DNA and virion-associated DNA present in culture supernatant were determined by qPCR analysis [13]. Total genomic DNA was extracted by using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). For extracellular viral DNA extraction, the supernatant (120 μl) was treated with 4 μl RQ1 DNase (Promega) for 1 h at 37°C, and reactions were stopped by adding 20 μl of stop buffer and incubation at 65°C for 10 min; 12.5 μl proteinase K (20 mg/ml, Invitrogen) and 25 μl 10% (wt/vol) SDS then were added to the reaction mixtures, which were incubated for 1 h at 65°C. DNA was purified by phenol-chloroform extraction followed by isopropanol-sodium acetate precipitation and then resuspended in 100 μl nuclease-free water. qPCR was performed as mentioned above. Relative levels of viral DNA were normalized to supernatant viral DNA without lytic induction. The BALF5 primers used for quantitating EBV copy numbers were described previously [13,105]. The reference gene β-actin was used for data normalization.

**In vitro caspase cleavage assay**

In vitro cleavage assay was performed as previously described [94]. Briefly, HA-tagged KAP1 was immunoprecipitated from transfected 293T cells using HA magnetic beads. The beads-bound HA-KAP1 and individual active caspases (active human caspases group IV; ApexBio,
Cat# K2060) were incubated in caspase assay buffer (50 mM HEPES, pH7.2, 50 mM NaCl, 0.1% Chaps, 10 mM EDTA, 5% Glycerol and 10mM DTT) at 37˚C for 2 hrs. Reactions were stopped by boiling in 2× SDS sample buffer and samples were analyzed by western blot.

**Immunoblot analysis**

Cell lysates were harvested in lysis buffer including protease inhibitors (Roche) as described previously [106]. Protein concentration was determined using the Bradford assay (Biorad), and proteins were separated in SDS 4–20% polyacrylamide gels and then transferred onto a PVDF membrane. Membranes were blocked in TBS containing 5% milk, and 0.1% Tween 20 solution. Membranes were then incubated in the following primary antibodies: mouse anti-ZTA (Argene, Cat # 11–007, 1:5,000), mouse anti-RTA (Argene, 1:1,000), mouse anti-BGLF4 antibody (1:1,000) [111], anti-β-actin (Sigma, Cat # A5441, 1:5,000), anti-IRF8 (CST, Cat #5628, 1:1,000), anti-PARP (CST, Cat #9532, 1:1,000), anti-Cleaved PARP (CST, Cat #5625, 1:1,000), anti-Cleaved Caspase Substrates (CST, Cat #8698, 1:1,000), anti-Caspase-1 (CST, Cat #3866, 1:1,000), anti-Caspase-2 (CST, Cat #2224, 1:1,000), anti-Caspase-3 (Santa Cruz, Cat #sc-7148, 1:1,000), anti-Cleaved Caspase-3 (CST, Cat #9664, 1:1,000), anti-Caspase-7 (CST, Cat #12827, 1:1,000), anti-Cleaved Caspase-7 (CST, Cat #8438, 1:1,000), anti-Caspase-8 (CST, Cat #9746, 1:1,000), anti-Cleaved Caspase-8 (CST, Cat #9496, 1:1,000), anti-Caspase-9 (CST, Cat #9508, 1:1,000), anti-Bcl-2 (Bethyl, Cat #A303-675A, 1:1,000), anti-KAP1 (CST, Cat #4123, 1:1,000), anti-PAX5 (CST, Cat #8970, 1:1,000), anti-DNMT3A (Bethyl, Cat #A304-278A, 1:1,000), anti-STAT3 (CST, Cat #9139, 1:1,000), and anti-HA (CST, Cat #14031S, 1:1,000). The secondary antibodies used were horseradish peroxidase (HRP)-labeled goat anti-mouse antibody (Fisher Scientific, 1:5,000) and HRP-labeled anti-rabbit antibody (Fisher scientific, 1:5,000).

**Bioinformatics analysis**

Potential caspase cleavage sites were searched for all the EBV protein sequences using Peptide-Cutter (http://web.expasy.org/peptide_cutter/) and the GraBCas software [96,97].

**Statistical analysis**

All numerical data were presented as mean ± standard deviation of triplicate assays. The statistical significances were determined using Student’s two-tail t-test, where p<0.05 was considered statistically significant.

**Supporting information**

S1 Table. A. The transcription level of all the genes identified in IRF8-sg2 and NC cell lines. B. Differentially expressed genes between IRF8-sg2 and NC cell lines.

S2 Table. The predicted caspase cleavage sites on EBV proteins.

S1 Fig. IRF8 depletion efficiency evaluated by Sanger sequencing. The sequencing of IRF8-depleted cell lines showing that 10 out of 22 clones for sg1 and 9 out of 14 clones for sg2 contain frame shifts. The PAM sequences were highlighted by red and the guide RNA sequences were shown in bold. WT: wild-type; “+” or “−” followed by numbers indicates the number of base pair inserted or deleted; “S” followed by numbers indicates the number of site mutations; “×” followed by numbers indicates the number of clones obtained in the sequencing.
**S2 Fig. IRF8 reconstitution facilitates EBV lytic replication upon lytic induction.** A. Akata (EBV⁺) IRF8-sg2 cells were used to establish IRF8-expressing stable cell lines using a pLX-IRF8 lentiviral construct. Western blot analyses showing IRF8, ZTA, RTA and BGLF4 expression level in different cell lines upon IgG cross-linking as indicated. B. Intracellular viral DNA from cells treated as in (A) was measured by qPCR using primers to EBV BALF5. The value of vector control at 0 hr (lane 4) was set as 1. Data are presented as means ± standard deviations of triplicate assays. **p<0.01. (TIF)**

**S3 Fig. IRF8 depletion suppresses caspase activation.** A. RT-qPCR validation of the 8 apoptosis-related genes in IRF8-sg1 cells. B and C. IRF8 depletion (sg1) suppresses caspase-1 expression and the generation of cleaved caspase substrates upon lytic induction by anti-IgG cross-linking. Western blot analysis of protein extracts from Fig 1C using antibodies against caspase-1, PARP, and cleaved caspase substrates as indicated in panel (B). Western blot analysis of protein extracts from Fig 1C using antibodies against caspase-3, cleaved caspase-3, caspase-8, cleaved caspase-8, caspase-7, cleaved caspase-7, caspase-9, cleaved caspase-9, caspase-2, Bcl2, KAP1, PAX5, DNMT3A and STAT3 as indicated in panel (C).

**S4 Fig. The relative expression level of CASPs in the control (NC) or IRF8-depleted (sg2) Akata (EBV⁺) cells obtained by RNA-seq analysis.** RPKM, Reads Per Kilobase of transcript per Million mapped reads.

**S5 Fig. IRF8 and IRF1 bind to CASP1 promoter and activate the promoter activity in B cells.** A. ChIP-PCR analysis using three EBV-positive cells [Akata (EBV⁺), P3HR-1 and LCLs] showing IRF8/IRF1 binding to CASP1 promoter. ChIP by a nonspecific IgG was include as negative controls. B. The pGL2-CASP1p-1-Luc constructs were co-transfected into Akata (EBV⁺) cells with either 10 ug of IRF8, IRF1 or IRF8-K108E expression vectors. Luciferase assays were performed 36 hrs post-transfection. The value of cells transfected with an empty vector was set as 1. The results were presented as mean ± standard deviation of triplicate assays. **p<0.01, ***p<0.001. (TIF)**

**S6 Fig. The relative expression level of IRFs in the control (NC) Akata (EBV⁺) cells obtained by RNA-seq analysis.** RPKM, Reads Per Kilobase of transcript per Million mapped reads.

**S7 Fig. CASP1 depletion efficiency evaluated by Sanger sequencing.** The sequencing of CASP1-depleted cell lines showing that 8 out of 13 clones for CASP1-sg1 and 12 out of 14 clones for CASP1-sg2 contain frame shifts. The PAM sequences were highlighted by red and the guide RNA sequences were shown in bold. WT: wild-type; "+" or "-" followed by numbers indicates the number of base pair inserted or deleted; "S" followed by numbers indicates the number of site mutations; "×" followed by numbers indicates the number of clones obtained in the sequencing.

**S8 Fig. CASP8 mRNA level upon CASP1 depletion.** qPCR analysis showing that CASP8 mRNA level was slightly increased by CASP1 depletion. The value was normalized by qPCR using specific primers to β-actin. Data are presented as means ± standard deviations of triplicate assays. (TIF)
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