Introduction

Epstein-Barr virus (EBV) is a member of the human gamma-herpesvirus subfamily that infects over 90% of the global adult population [1,2]. EBV preferentially establishes latent infection in B-lymphocytes but can also infect epithelial cells [3,4]. EBV primary infection is one of the main causes of infectious mononucleosis (IM); while EBV latent infection is associated with multiple malignancies such as nasopharyngeal carcinoma, Burkitt’s lymphoma, and Hodgkin’s lymphoma [3,4]. Furthermore, EBV is responsible for the majority of lymphoproliferative diseases associated with AIDS and immunosuppression following organ transplant [5]. Like all herpesviruses, EBV exists in a dynamic balance between productive and latent infection. The factors that regulate the fate decisions for lytic reactivation from latency have been investigated in some detail, but relatively little is known about the fate regulation during the earliest stages of primary infection. Upon entry into the nuclear compartment, herpesvirus DNA genomes must confront several intrinsic anti-viral resistances that restrict viral gene expression and replication. One prominent nuclear structure involved in antiviral resistances is the PML nuclear body (PML-NB), also referred to as nuclear domain 10 (ND10). PML-NBs are nuleoplasmic protein aggregates mainly consisting of (but not limited to) the components PML, Sp100, Daxx, and ATRX [6,7]. The size and abundance of PML-NB is interferon inducible [8,9,10], and over-expression of the PML protein represses viral infection [11]. PML-NB is the nuclear localization site of many DNA viruses, including Herpes Simplex virus (HSV-1), Human Cytomegalovirus (HCMV) and Adenovirus (Ad5) [12,13]. These viruses then modify the morphology and/or protein composition of PML-NBs shortly after infection [12,14]. The mechanism of PML-NB-mediated antiviral repression is not clearly determined. PML, Sp100, and Daxx are all associated with transcription repression, and this function may act on viral genomes [15]. Daxx can act as a transcription co-repressor of many cellular transcription factors [16,17,18,19], and forms repressive transcription complexes with histone deacetylases (HDACs) [20,21] and DNA methyltransferase I (DNMT I) [22,23]. Daxx has been shown to induce heterochromatin markers on the HCMV genome and repress viral gene expression in a HDAC dependent manner [24,25]. Daxx also forms a chromatin-remodeling complex with ATRX [26] and both can form a repression complex at heterochromatin [27]. Furthermore, RNA interference (RNAi) studies have shown that knockdown of Daxx or ATRX can result in a higher infection level of HCMV [28,29,30] and also relieve the infection defect of mutant HSV deficient in disrupting PML-NB [31]. Herpesviruses confront intrinsic anti-viral resistances immediately upon entering the host cell nucleus, and therefore must counteract these resistances at the earliest possible time points to...
Author Summary

Persistent infection by Epstein-Barr virus (EBV) is associated with a variety of diseases, including lymphoid and epithelial tumors. Despite a wealth of information on the mechanism of viral persistence, relatively little is known about the early steps of EBV infection and viral gene activation. Host cells actively mount resistances against viral infection, which viruses need to overcome to invade the cell. We have found that among the proteins packaged in the EBV viral particle, BNRF1 plays an important role of counteracting cellular defenses. We show that EBV protein BNRF1 binds to the cellular protein Daxx and disassembles the Daxx-ATRX complex, where both Daxx and ATRX are cellular proteins known to inhibit viral gene expression. We also confirm that BNRF1 can promote expression of early viral genes, and that Daxx-binding by BNRF1 is required for this function. Finally, we demonstrate that Daxx and ATRX repress viral gene expression during latency. We conclude that BNRF1 disassembles cellular antiviral defense machinery to promote expression of viral genes in the host cell.

initiate viral gene expression. Herpesvirus tegument proteins, which are pre-packaged and delivered with the infectious virion, are strategically positioned to counteract the intrinsic anti-viral defenses and support the early steps of infection [32]. Both alpha- and beta-herpesviruses encode tegument proteins that regulate early events during lytic replication, including the disruption of the PML-NBs. HSV-1 immediate early gene ICP0, disrupts PML-NB structure by degrading the core component PML [33,34,35] and eliminating SUMO-modified Sp100 [36]; while HCMV tegument protein pp71 displaces ATRX and subsequently degrades Daxx [24,30]. Both ICP0-deficient HSV-1 and pp71-deficient HCMV mutants are deficient in infection, where viral gene expression is shutdown, resulting in a dormant viral genome [29,37,38]. Interestingly, it has been reported that disruption of PML-NB by ICP0 is mediated by de novo synthesized ICP0, instead of tegument delivered ICP0 protein, suggesting that this event is coordinated with early viral gene activation or, perhaps, reactivation from latent infection [35]. We have previously shown that EBV genomes localize to and then disrupt PML-NB during lytic replication; while latent EBV episomes are segregated away from PML-NBs during latency [39]. EBV regulatory proteins, including the lytic cycle immediate early gene Zta (also referred to as BZLF1, ZEBRA, and Z), and latency associated EBNA1 and EBNA-LP, have been implicated in PML-NB interactions [40,41,42]. However, it remains unclear if PML-NBs regulate early events associated with viral gene expression upon EBV nuclear entry, and if an EBV tegument protein modulates this intrinsic defense.

The EBV major tegument protein BNRF1 is one of the most abundant tegument proteins in the virion [43] and is essential for the establishment of viral latent infection [44], yet its function is largely unknown. BNRF1 homologues are present in all gammaherpesviruses but absent in the alpha- and beta- herpesvirus subfamilies. All BNRF1 orthologues share regions homologous to the cellular enzymes Phosphoribosylformylglycinamidine Amidotransferase (FGARAT) and Aminoimidazole ribonucleotide (AIR) synthetase, ATP-dependent enzymes in the 4th and 5th steps of the purine de novo biosynthesis pathway. However, no enzymatic activity has been found in any BNRF1 orthologues. In a knockout study, transfected BNRF1-deficient EBV genomes can reactivate from latency, produce morphologically normal virions, and the progeny can enter cells with little observed defects [44]. Yet, upon infection of B cells the mutant virus showed a 20-fold lower expression of a viral latency associated gene EBNA2 and failed to induce B cell transformation [44]. This suggests an important role of BNRF1 in supporting early infection. Furthermore, the BNRF1 orthologue encoded by murine herpes virus 68 (MHV68), tegument protein ORF75c, induces PML degradation and is essential for initiation of viral gene expression [45,46].

Here, we demonstrate that EBV BNRF1 is a novel PML-NB-interacting viral protein, and that this interaction is important for supporting EBV primary infection. We first show that Daxx is a primary cellular interaction partner of BNRF1. BNRF1 co-localizes with Daxx at PML-NB foci while disrupting the Daxx-ATRX complex. Furthermore, we identify a novel Daxx interaction domain on BNRF1. This domain is essential for BNRF1 to interact with Daxx, localize to PML-NB, and displace ATRX from Daxx. We then show that BNRF1 supports EBV primary infection and promotes the expression of viral genes soon after viral genomes enter the cell, and that the Daxx interaction domain contributes to these functions. Lastly, we show that knockdown of either Daxx or ATRX results in disruption of viral latency, suggesting that Daxx and ATRX play a role in the restriction of viral gene expression. Our study suggests that EBV tegument protein BNRF1 disassemble the Daxx-ATRX antiviral resistance complex to enable viral gene expression after cell invasion, and likely regulate the chromatin organization for the establishment of latent infection.

Results

BNRF1 interacts with cellular protein Daxx

To characterize the biological properties of the EBV major tegument protein BNRF1, we took a proteomic approach to screen for potential cellular interaction partners. BNRF1 was cloned into a 3x FLAG tag expression vector under the control of a CMV promoter. 293T cells were then stably transfected with either FLAG-vector or FLAG-tagged BNRF1. Nuclear extracts from stable cell lines were subject to immunopurification (IP) with a FLAG antibody, and then analyzed by SDS-PAGE (Fig. 1A). Bands unique to the BNRF1 lane (B) were cut out and analyzed by liquid chromatography-tandem mass spectrometry (LC/MS/MS). The major identified species was BNRF1, but substoichiometric proteins enriched in the BNRF1 IP were also identified, including Daxx, nucleophosmin (NPM1), and PARP1 (Fig. 1C). We subsequently confirmed in BNRF1 transiently transfected 293T cells that Daxx co-precipitates with BNRF1 (Fig. 1B) by both FLAG pull-down and the Daxx reverse pull-down, indicating a stable in vivo interaction between BNRF1 and Daxx. Neither PARP1 nor NPM1 interaction with BNRF1 could be validated by subsequent co-IPs (data not shown), we therefore focused our efforts on characterizing the interaction with Daxx.

BNRF1 utilizes a novel Daxx interaction domain

To further characterize the interaction between BNRF1 and Daxx, we introduced serial deletions on the FLAG-BNRF1 expression plasmid. We first made five deletion constructs of BNRF1, sequentially deleting regions coding for 300 amino acids (Fig. 2A, constructs d1 through d5). We then performed IPs with Daxx, we introduced serial deletions on the FLAG-BNRF1 expression plasmid. 293T cells were then stably transfected with FLAG, or Daxx on lysates of cells...
interacting with Daxx, we conclude that a putative Daxx-interaction domain is located in the region between 300–600aa of BNRF1.

We then further made six serial deletions of 60 amino acids in the 300–600 aa region (Fig. 3A, constructs d21 through d26) to narrow down the suspected Daxx-interaction domain to a smaller region. After a subsequent round of IP pull-downs, we found that all BNRF1 deletions, with the exception of d21, were defective in binding Daxx (Fig. 3B), suggesting that the 360–600 aa region of BNRF1 is responsible for interaction with Daxx. To determine if this region was sufficient for interaction with Daxx, we expressed only the 300–600 aa region in the FLAG-expression vector (Fig. 3A, construct DID) and performed IP pull-downs. We found that this region bound Daxx as efficiently as WT-BNRF1, in both FLAG IP and in the reverse IP with anti-Daxx antibody (Fig. 3C). Notably, we failed to find sequence homology of this Daxx interaction domain with any known protein motif, and this domain is also distinct from the FGARAT and AIR synthetase homology regions, efficiently pulled down both ATRX and Daxx. The d1 and d5 truncations, which lie outside of the FGARAT and AIR synthetase homology regions, pulled down only Daxx but not ATRX, suggesting it efficiently disrupted the ATRX-Daxx interaction similar to WT. These data suggest that the FGARAT and AIR synthetase homology regions of BNRF1 may contribute to the disruption of ATRX-Daxx complex.

**BNRF1 disrupts the Daxx-ATRX chromatin remodeling complex**

Daxx forms a chromatin remodeling complex with ATRX [26] and ATRX has been implicated in the transcriptional repression of both HSV-1 and HCMV during the early steps of infection [30]. Moreover, both HSV-1 and HCMV utilize viral encoded enzymes Aminoimidazole ribonucleotide synthetase (AIR) and Type 1 glutamine amidotransferase (GATase1, an enzymatic domain of FGARAT), as identified by the National Center for Biotechnology Information (NCBI) conserved domain search. (B) IP pull down analysis of the BNRF1 deletion constructs. 293T cells were either transfected with empty FLAG vector (V), the BNRF1 constructs WT, or mutants d1-d5. Cell lysates of transfected cells were then subject to IP pull-downs with non-specific IgG, anti-FLAG, or anti-Daxx- antibodies, then Western blots were probed for Daxx (top panels), and FLAG-tagged proteins (lower panels). Input is shown for each mutant in the left most panels. doi:10.1371/journal.ppat.1002376.g002

**BNRF1 co-localizes with Daxx to PML nuclear bodies and disperses ATRX from nuclear bodies, in a Daxx interaction domain-dependent manner**

Daxx is a prominent component of PML nuclear bodies [47], and Daxx localization at these nuclear bodies are disrupted by viral proteins of both HSV-1 and HCMV [24,34]. Thus, it is
important to investigate the sub-cellular location of BNRF1-Daxx interaction, and check if BNRF1 disrupts Daxx-ATRX localization in the nuclear bodies. For immunofluorescence (IF) microscopy studies, we selected Hep2 carcinoma cell lines because of their larger size and prominent PML nuclear bodies, and their common use in many previous studies with herpesvirus protein interactions with PML-NBs. Hep2 cells were transiently transfected with empty FLAG vector (V) or BNRF1 constructs WT, d26, or DID. Cells were then fixed two days post transfection and subjected to IF staining. We found that WT BNRF1 partially co-localized with foci containing Daxx (Fig. 4A, S1A and Table S1), PML (Fig. 4B, S1B and Table S1), and Sp100 (Fig. S1D), suggesting that BNRF1 interacts with Daxx at the PML nuclear bodies. We also noticed that DID itself is sufficient for localizing to PML nuclear bodies, while the d26 deletion mutant showed a weak dispersed pattern in the cell (Fig. 4A and B, S1A and Table S1). The co-localizations were also confirmed by line scan analysis, where the BNRF1 WT and DID intensity peaks overlap with Daxx and PML peaks (Fig. S2). To ensure that the diffuse pattern of the d26 mutant is not due to deficient protein expression, the same set of transfected cells as used for IF were also assayed by Western blot for total expression levels of BNRF1 proteins (Fig. 4D). We found that d26 protein was expressed at levels similar to that of WT, despite its diffuse staining in IF studies, confirming its protein expression in the cells used in our microscopy study. These findings indicate that the interaction with Daxx is necessary and sufficient for BNRF1 to localize to the nuclear bodies.

To understand the BNRF1 disruption of Daxx-ATRX complex in a sub-cellular spatial context, we also examined ATRX by IF in BNRF1-transfected Hep2 cells (Fig. 4C and S1C). Again, we found ATRX foci co-localizing with WT BNRF1 and DID but not d26, which is also confirmed by line scan analysis (Fig. S2C). However, we also found a substantial reduction in ATRX foci intensity when cells were transfected with WT BNRF1, but no apparent reduction when transfected with d26 or DID-mutants (Fig. 4C). The failure of DID to disperse ATRX is consistent with its only partial disruption of ATRX from Daxx IP (Fig. 3C). Quantification of Daxx (Fig. 4E), PML (Fig. 4F), and ATRX (Fig. 4G) nuclear foci in BNRF1-expressing cells compared to non-expressing cells revealed that BNRF1-expressing cells contain a significantly lower (p<0.0001) average number of ATRX nuclear foci than non-expressing cells (Fig. 4G). In contrast, we found no significant difference in the number of Daxx (Fig. 4E) and PML nuclear foci (Fig. 4F) in BNRF1 transfected cells. Taken together, these results suggest that BNRF1 not only disrupts the Daxx-ATRX complex, but also actively disperses ATRX away from nuclear bodies.

HSV-1 ICP0 and HCMV pp71 each induce the degradation of PML and Daxx proteins respectively, yet we did not observe any evidence of this with BNRF1 in our microscopy studies. To investigate the potential degradation of PML, Daxx and ATRX proteins by BNRF1, we examined the stability of these proteins in BNRF1 stably transfected cells (Fig. 5A). 293T cells stably transfected with control vector (clone C) or WT BNRF1 (stable transfection clones 3 and 9) were lysed and subjected to Western blot analysis. We found no evidence of degradation or gross post-translational modification of PML, Daxx, nor ATRX in BNRF1-expressing cell lines. We also analyzed the protein stability of PML and Daxx in Hep2 cells transiently transfected with control vector, WT BNRF1 or d26, and again found no evidence of BNRF1-induced protein degradation (Fig. S3). This suggests that BNRF1 does not mimic the protein degradation function of HCMV pp71 or HSV-1 ICP0, but rather, disrupts Daxx-ATRX interactions through alternative mechanisms.

The diffuse distribution of the BNRF1-d26 mutant raised the question of whether the Daxx interaction domain of BNRF1 correlated with nuclear localization. To test this, we utilized biochemical fractionation methods to isolate nuclear and cytoplasmic proteins from 293T cells (Fig. 5B). We found that WT
BNRF1 localized to both cytoplasmic (~60%) and nuclear (40%) fractions. The d26 mutant, which is deficient in both Daxx interaction and nuclear bodies localization, was expressed at lower amounts yet showed a cytoplasmic to nuclear distribution similar to WT (Fig. 5B). This is consistent with d26 having a weak diffuse nuclear and cytoplasmic staining in IF (Fig. 4). Meanwhile, the
Daxx-ATRX Disruption by EBV Tegument BNRF1

DID mutant, which binds Daxx and co-localizes with PML nuclear bodies in the nucleus, was isolated at low, yet detectable levels in the nucleus; although substantially more was recovered in the cytoplasmic fraction. The efficiency of the fractionation was confirmed by the presence of PARP1 exclusively in the nuclear fraction. Transfected cells were assayed by Western blot with antibody to FLAG (BNRF1), PARP1 (nuclear marker) or α-Tubulin (cytoplasmic marker).

The Daxx interaction domain is required for BNRF1 to support EBV primary infection

A previous study using an EBV bacmid with a BNRF1-knockout demonstrated that BNRF1-mutant virions can be generated from producer 293 cells and can enter the cytosol of infected B-cells; yet mutant virus failed to express one of the first expressed latent genes, EBNA2, upon primary infection of B cells, and were incapable of inducing B-cell proliferation [44]. To understand the role of BNRF1-Daxx interaction in primary infection, we took a complementation rescue approach with the BNRF1-mutant virus. 293 cells stably transfected with either wild type or BNRF1-knockout EBV bacmids were used for virus production (Fig. 6). As the EBV bacmids also encode GFP, cells infected with this bacmid-derived virus could be visualized by the presence of green fluorescence. To induce viral production, we reasoned that reporter plasmids may lack essential BNRF1 regulation, we first tested the effect of BNRF1 on reporter plasmids using transient transfection assays, but found no consistent effect on candidate viral promoters (data not shown).

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To investigate the potential mechanism of BNRF1 in viral gene regulation, we first tested the effect of BNRF1 on reporter plasmids using transient transfection assays, but found no consistent effect on candidate viral promoters (data not shown). We reasoned that reporter plasmids may lack essential BNRF1 target elements or chromatin assembly, and therefore assayed BNRF1 activity on EBV bacmid genomes after transfection into 293 cells (Fig. 7B). EBV bacmid DNA (Bac36) and either empty FLAG-vector, WT BNRF1 or d26 mutant BNRF1 were co-transfected into 293 cells and assayed 3 days post transfection for viral gene expression using RT-qPCR. We found that WT BNRF1 complementation induced an up-regulation of EBNA1, EBNA2 and BZLF1 mRNA expression compared with non-complemented virus or the d26-mutant complementation. Interestingly, background levels of BZLF1 expression were detectable in non-complemented and d26 mutant infections, suggesting that BNRF1 may only partly enhance BZLF1 expression, which can occur at low levels independently of BNRF1.

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BNRF1 mutant virus infection can be rescued by ectopic complementation with wild type, but not Daxx-interaction-deficient BNRF1. Primary B cells were infected with equal volumes of GFP expressing virus, either produced from wild type bacmids or ΔBNRF1 mutant virus complemented with empty FLAG vector, wild type, or the d26 Daxx-interaction-deficient mutant BNRF1. Infected cells would express the GFP carried in the virus. Infection rate as shown in fluorescent microscopy photos (A), or a manual count of the average number of GFP-positive cells per well (B). Aliquots of the virus used for infection were isolated for measurement of viral titers by reverse transcription PCR assay of viral gene expression for EBNA1, EBNA2, or BZLF1. (B) 293HEK cells were co-transfected with wild type EBV genomes and either empty vector, WT BNRF1 or the d26 Daxx-interaction-deficient mutant BNRF1. Virus infected cells were subject to reverse transcription PCR assay of viral gene expression for EBNA1, EBNA2, EBN3C, or BZLF1.

Discussion

The specific class of antiviral defense dubbed the intrinsic immunity [51,52] plays a broad and general role in restricting viral infection. PML-NBs and its associated proteins such as PML, Sp100, Daxx and ATRX, have been extensively studied as cellular defenses against herpesviruses, specifically with the alphaherpesvirus HSV-1 and betaherpesvirus HCMV. Upon the early stages of infection right after cell entry, HSV-1 and HCMV utilize viral proteins that effectively disrupt the structure and disable the function of the PML-NBs in restricting viral gene expression and replication. However, the gammaherpesvirus EBV has been relatively less studied in terms of how it counteracts these cellular defenses upon primary infection or reactivation. We show here that the major tegument protein of EBV, BNRF1, interacts with Daxx (Figs. 1–3, S1–2) and disrupts its ability to form a complex with ATRX or recruit ATRX to PML-NBs (Figs. 3–4, S1–2). The disruption of the Daxx-ATRX complex contributes to viral gene expression from latently infected B-cells, and suggest that BNRF1 disruption of the Daxx-ATRX complex contributes to viral gene control during early infection and reactivation.
BNRF1 and pp71 share no obvious amino acid sequence similarity, and the Daxx interaction domains of these two proteins vary significantly in amino acid composition and size of the interaction domains. These findings suggest that BNRF1 is a functional homologue of pp71, but utilizes a distinct mechanism for the dissociation of ATRX from PML-NBs.

Herpesvirus tegument proteins have been implicated in the determination of viral lytic or latent gene expression programs. Restriction of tegument protein entry into the nucleus, as has been shown for HCMV pp71 and HSV VP16, correlates with the establishment of latency [24], suggesting that tegument proteins may play a critical role in determining lytic or latent gene expression programs. Interestingly, we found that the Daxx-interaction deficient BNRF1 mutant d26, which fails to interact with Daxx, showed a weak diffuse subcellular distribution instead of the punctate nuclear dots of WT BNRF1 (Fig. 4 and S1). Similarly, biochemical fractionation studies (Fig. 5B) suggest that while BNRF1 can localize to both the cytoplasm and nucleus, it may require the Daxx-interaction domain to efficiently accumulate in the nucleus. Potentially related is the observation that HCMV pp71 translocation to PML-NBs is also dependent on its interaction with Daxx [54]. Selective cytoplasmic retention of several herpesvirus tegument proteins, including pp71 and VP16, may play a critical role in determining lytic or latent gene expression programs [55]. We suspect that BNRF1 might be subject to similar regulation through its PML-NB localization.

Daxx and ATRX are known to play a global role in the control of cellular and viral gene expression and chromosomal structure. Daxx itself has been shown to associate with HDACs and to function as a global repressor of transcription [20, 21]. The Daxx-ATRX complex has in vitro chromatin remodeling activities [26] and can function as a histone H3.3 chaperone [56]. Recent studies suggest also that ATRX interacts with G-rich repeat chromatin regions [57], and in collaboration with Daxx load histone variant H3.3 onto pericentromeric and telomeric chromatin [56, 58, 59]. H3.3 is generally associated with open chromatin and active transcription when loaded by the histone chaperoneHIRA [60]. However, the Daxx-ATRX complex loaded H3.3 has been found to facilitate transcription from pericentromeric regions [58] but repress transcription from telomeric regions [59]. Interestingly, HIRA-loaded H3.3 can facilitate the lytic replication of HSV-1 during the early steps of infection [61]. Furthermore, the Daxx-degrading pp71 blocks the establishment of heterochromatin on the HCMV Major Immediate Early Promoter (MIEP) region [25]. These findings underscore the importance of host chromatin regulatory mechanisms in the control of herpesvirus infection. We suspect that the viral gene activation function of BNRF1 (Fig. 7) is likely to be mediated by chromatin-dependent processes since we failed to observe consistent transcription activation when assayed in transient plasmid-based reporter assays using EBV promoters for BZLF1 (Zp) or EBNA2 (Cp or Wp) (data not shown). We propose that BNRF1 stimulates EBV early gene activity through de-repression of the Daxx-ATRX mediated chromatin repression mechanism, perhaps similar to that of pp71 de-repression of the HCMV MIEP locus. However, the precise molecular mechanism through which BNRF1 activates early gene transcription remains to be investigated.

While not explored yet, it is also not known if the FGARAT enzyme-homology domain of BNRF1 has any function in the context of supporting viral infection. This enzyme homology is conserved among all gammaherpesvirus orthologues of BNRF1, including the KSHV and MHV68 ORF75 family members. Despite significant sequence similarity with BNRF1, KSHV and
supplemented with 2.5 clone C were grown in DMEM as described for 293T cells above, FLAG-BNRF1 (clone 3 and clone 9) and empty FLAG vector

Human B Cell isolation kit (Invitrogen). All cells were grown in a Ficoll-Paque Plus purchased from GE healthcare. Primary B cells donated human blood by density gradient centrifugation with Hep2 and 293T cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20 mM GlutaMAX (Gibco), 100 U/ml penicillin and 100 µl/ml streptomycin. 293HEK cells were grown in minimum essential medium Eagle (MEM), supplemented with 10% FBS and 20 mM GlutaMAX (Gibco). DG75 and Mutu I cells are EBV

Materials and Methods

ethics statement

Human B-lymphocytes were obtained from the Wistar Institute phlebotomy lab. All samples were from anonymous adult donors and approved by the Wistar Institute Institutional Review Board. Written informed consent was provided by study participants.

Cells

Hep2 and 293T cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20 mM GlutaMAX (Gibco), 100 U/ml penicillin and 100 µl/ml streptomycin. 293HEK cells were grown in minimum essential medium Eagle (MEM), supplemented with 10% FBS and 20 mM GlutaMAX (Gibco). DG75 and Mutu I cells are EBV negative and positive (respectively) Burkitt’s lymphoma cell lines, grown in RPMI 1640 medium supplemented with 10% FBS, and approved by the Wistar Institute Institutional Review Board. Written informed consent was provided by study participants.

Viruses bacmids and virus production cells

Viruses were produced using chloramphenicol and hygromycin resistant bacmids containing the EBV genome and the gene coding for green fluorescence protein (GFP). 293/EBV-wt cells (a gift from H. J. Delecluse) are 293HEK cells stably transfected with the wild type EBV bacmid [62]. 293/ΔBNRF1 cells (a gift from H. J. Delecluse) are 293HEK cells stably transfected with an EBV bacmid with the BNRF1 gene deleted [44]. 293/EBV-wt and 293/ΔBNRF1 cells were grown in RPMI 1640 medium supplemented with 10% FBS and 100 µg/ml hygromycin.

Enzymes and antibodies

All restriction enzymes, T4 DNA ligase and associated buffers were purchased from New England Biolabs. Monoclonal mouse anti-FLAG antibody (F1804), Polyclonal rabbit anti-FLAG antibody (F7425), Polyclonal rabbit anti-Daxx antibody (F7810), Monoclonal Anti-α-Tubulin antibody (T5168), Monoclonal mouse anti-β-Actin-Peroxidase antibody (A3854), and Anti-mouse IgG R-Phycoerythrin (PE) conjugated antibody (P8547) were purchased from Sigma-Aldrich. Monoclonal mice anti-pML (PG-M3, sc-966), and polyclonal rabbit anti-ATRX (H-300, sc-15408), and polyclonal rabbit anti-ZEB1 (sc25388) were purchased from Santa Cruz Biotechnology. Polyclonal rabbit anti-PARP1 antibody (ALX-210-895-R100) was purchased from Enzo Life Sciences. Mouse anti-EA-D antibody was purchased from Millipore. Anti-EBV-VCA (0231) antibody was purchased from Pierce Thermo Scientific.

Construction of BNRF1 expression plasmids and truncations

BNRF1 was cloned into the HindIII-Sall sites of the p3xFLAG-Myc-CMV-24 Expression Vector (Sigma-Aldrich), using the PCR primers: gcgaagcttgaagagagggcagggaaacgcaa and gcggtcgactcactgagggagggccggtg. BNRF1 deletion mutants were generated as follows. PCR Primers (Table S2) were designed so that the front and rear halves of the DNA oligo each binds the 5’ or 3’ regions flanking the targeted deletion site on the BNRF1 template. PCR reactions were setup using iProof High-Fidelity DNA polymerase 2x master mix (Bio-Rad), with primers at 1 μM concentration, and the FLAG-BNRF1 expression plasmid as the template at a concentration of 30 ng DNA in a 25 μl reaction setup. PCR was done with a Bio-Rad C1000 thermal cycler, thermal cycles setup according to DNA polymerase mix manufacturer suggested conditions. To clear out the wild type BNRF1 template, 15 μl of the PCR product were treated with 30 U DpnI [New England Biolabs] in a 20 μl reaction for 2 hours to over night at 37°C. 2 μl of DpnI-treated DNA were then transformed into 50 μl of Library Efficiency DH5α competent cells (Invitrogen). Colonies were screened for the deletion by enzyme digestion analysis of miniprep DNA, and then confirmed by DNA sequencing of the expected deletion site.

Immunoprecipitation

BNRF1 expression plasmids were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer instructions. Cells were harvested 2 days post transfection by washing cells off the plate with PBS. Harvested cells were further washed 3 times with cold PBS, and then subject to lysis with freshly prepared NET lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and 0.1% mammalian protease inhibitor cocktail mix [P8340, Sigma-Aldrich], at 1 ml NET per IP pulldown. Cell lysates were homogenized by doing 10 strokes in a Dounce homogenizer. 60 µl of each lysate were isolated after this step as input control. The remaining lysates were incubated at 4°C rotating for 30 mins to fully solubilize proteins. Lysates were then spun at 13000 rpm 5 mins to remove insoluble cell debris, then antibodies were added (5 µl of each antibody per IP) to the cleared lysates, and left rotating over night. 100 µl of 50% slurry of Protein A sepharose beads (GE healthcare) in NET buffer was added to each IP with rotating at 4°C for 2–3 hours, then washed three times with NET for 10 mins (rotating at 4°C) per wash. Pulled
down proteins were released by adding 50 μl 2x Laemmli buffer (100 mM Tris-Cl pH 6.8, 4% SDS, 0.2% Bromophenol Blue, 20% Glycerol), and boiling for 10 mins at 100°C. The resulting samples (excluding beads) were then loaded directly into protein gels and subject to Western blot analysis.

For mass spectrometry identification of BNRF1 associated proteins, FLAG-BNRF1 expressing and FLAG-vector control stable cell lines were generated as mentioned above. Nuclear extracts from 5×10⁷ cells were subject to immunopurification with anti-FLAG Sepharose beads (A2220, Sigma-Aldrich) followed extensive washing with NET buffer, and FLAG peptide elution. Eluted protein was subject to precipitation with 10% trichloroacetic acid (TCA) followed by SDS-PAGE and colloidal blue staining. Sections of the gel with enriched polypeptides were subject to LC/MS/MS at the Wistar Proteomics Facility.

Immunofluorescence microscopy
Hep2 cells were transfected with BNRF1 expression plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, transfected cells were then reseeded at 2.7×10⁵ cells/well in 24 well plates containing microscope coverslips 5 hours post transfection. 2 days post transfection, coverslips with cells attached were harvested, fixed with 1% paraformaldehyde at room temperature for 15 mins, then permeabilized with 0.5% Triton-X 100. Coverslips were then stained with the first antibodies overnight at 4°C. First antibody dilutions used were as follows: mouse anti-FLAG at 1:20000, rabbit anti-Daxx at 1:5000, rabbit anti-FLAG at 1:5000, mouse anti-PML at 1:250, rabbit anti-ATRX at 1:250, all antibodies diluted in PBS. Second antibody stainings were carried out for 1 hour at room temperature with the red-fluorescent Alexafluor594 goat anti-rabbit antibody and green Alexafluor488 goat anti-mouse antibody (both from Invitrogen) each at 1/800 dilution in PBS. Coverslips were washed twice in PBS for 5 mins between each of the above treatments. Cell nuclei were stained briefly with DAPI (diluted to a final concentration of 0.167 μg/ml in PBS) for 2 mins, then washed with PBS, 70% EtOH, then 100% EtOH to wash out residual salts. Coverslips were air-dried briefly, and then mounted onto microscope slides with Vectashield mounting media (Vector Laboratories). Mounted slides were studied using a Nikon E600 upright microscope with a 100x oil objective. Photos for nuclear body quantification were taken using a 40x objective to maximize the number of cells in each photo while retaining a clear view of PML bodies.

Nuclear body quantification
Microscopy photos were analyzed using ImagePro Plus 6.2 software (Media Cybernetics). Photos were pre-processed by subtracting out the background intensity using the operation function (with a value of ~30), and passing through a flatten filter (a value of 10). A morphological ‘top hat’ filter was then applied to emphasize points or grains brighter then the background. The number of nuclear bodies in each cell nucleus was counted by quantifying the object numbers after applying the signal intensity threshold/segmentation tool to select the nuclear bodies as objects. Cell boundaries were defined by the outline from DAPI channel photos of the same field, while omitting all cells on the border of the image border. Resulting quantification numbers were then analyzed using Prism 4 software (Graph Pad Software), statistical analysis did by Mann-Whitney U non-parametric, unpaired t test.

Subcellular fractionation assay
293T cells were transfected in 10 cm plates with 2 μg expression plasmids of either empty FLAG vector, WT-BNRF1, or 6 μg of BNRF1-d26. Transfection was carried out using 10 μl Lipofectamine 2000 (Invitrogen) per transfection, following manufacturer instructions. Cells were harvested 24 hrs post transfection. 1/6 of cells isolated as input control. The rest of the cell pellets were fractionated with the Fermentas ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit (K0311). The resulting cytoplasmic and nuclear fractions, along with the input samples, were analyzed by Western blot.

BNRF1 complementation virus infection assay
To induce lytic virus production, 293/EBV-*rt and 293/ΔBNRF1 cells were transfected in 10 cm plates with expression plasmids of 1.75 μg BZLF1, 3.25 μg BALF2, or cDNA of empty vector, and 3 μg of either empty FLAG vector or 3 μg BNRF1 or 7.5 μg BNRF1-d26. Transfection was carried out using 15 μl Lipofectamine 2000 (Invitrogen) per transfection, following manufacturer instructions. The media of virus production cells were harvested 3 days post transfection, filtered through 0.45 μm filters, and added directly to freshly isolated primary B cells. B cells in virus containing media were centrifuged for 1200 rpm 90 mins at 25°C to enhance infection.

For measuring infection by GFP levels, infected B cells were treated with 1 mM Sodium Butyrate and 20 ng/ml TPA 3 days post transfection to enhance GFP expression, and the number of GFP positive cells in each well were counted manually under a Nikon TE2000 microscope using a 20x objective.

For measuring virus gene expression in infected B cells, cells were collected 4 days post transfection, and total RNA was purified using Trizol (Invitrogen). The resulting RNA was then subject to DNase 1 treatment at 2 U/50 μl, 1 hour at 37°C, then DNase was heat inactivated by adding a final concentration of 5 mM EDTA and incubated at 70°C for 10 mins. cDNA was synthesized using the Super Script III first strand synthesis system reverse-transcription kit (Invitrogen). The resulting cDNA was then subject to real time PCR analysis by ΔΔCt method and normalized to viral titers, measured as described below. Real time PCR primers used are listed in Table S3.

To measure the amount of complemented BNRF1 protein that were packaged into virions, ΔBNRF1 virions complemented with WT-BNRF1, BNRF1-d26, or un-complemented, were produced as mentioned above. 100 μl of the harvested and filtered virus-containing media were isolated for viral titer measurement as described below. The rest of the virus-containing media were concentrated by loading the media above a 5 ml layer of 22.5% sucrose in PBS, then centrifuged at 27000 rpm (~100,000) g 4°C for 1 hour in a SureSpin 630 Rotor (Thermo Scientific) with a Sorvall WX 100 Ultra ultracentrifuge. The resulting virus pellet was then resuspended in PBS, and analyzed by Western blot. Protein gel loading volumes were normalized according to viral titers to ensure equal amounts of virion protein in each well.

Virus titer measure by real time PCR detection of DNA copy number
Viral DNA in media was extracted as described by C. Busse et al. [63]. Virus-containing media were treated with 5 U/50 μl of DNase I (New England Biolabs) for 1 hour at 37°C. DNase was then deactivated by adding EDTA to a final concentration of 5 mM, followed by 10 mins heat inactivation at 70°C. Samples were then mixed 1:1 with 0.1 mg/ml of proteinase K in water, and incubated at 50°C for 1 hour, followed by 20 mins of heat inactivation at 75°C. The released viral DNA was measured by real time PCR analysis, using a serial dilution series of Namalwa cell lysate as the standard curve, which contain two copies of integrated EBV genome per Namalwa cell. EBV genomes were
detected using primers specific to the OriLyt region: 5′-CCTGTCTTACTGCCAGCTTACTG-3′ (OriLyt-fwd), 5′-AGTGAGGAGGCCAGAAATG-3′ (OriLyt-rev).

**Virus gene expression assay on bacmid/BNRF1 co-transfected cells**

Wild type EBV genome bacmids were prepared from 2.5 ml overnight LB culture using the Bacmax DNA purification kit (Epicentre). 293HEK cells were seeded (2.3 million cells per plate) the previous day in 10 cm plates, and transfected with 1.5 μg freshly prepared bacmids along with 0.5 μg of either empty FLAG vector, BNRF1, or BNRF1-d26 mutant. Transfection was carried out using Effectene transfection reagents (Qiagen), following manufacturer instructions. Cells were harvested three days post transfection, total RNA was purified using Trizol (Invitrogen), and then subject to DNase treatment at 2 U/50 μl, 1 hour at 37°C. DNase was heat inactivated by adding a final concentration of 5 mM EDTA and incubated at 70°C for 10 mins. cDNA was then synthesized using the Super Script III First Strand synthesis system reverse-transcription kit (Invitrogen). The resulting cDNA was then subject to real time PCR analysis by ΔΔCt method. Real time PCR primers used are listed in Table S3.

**shRNA-mediated knockdown of genes**

shNeg (pLKO-shNeg), shDaxx (pLKO-shDaxx-2) and shATRX (pLKO-shATRX200) constructs in lentivirus production plasmid backbones were generous gifts from Roger Everett. shNeg (sequence TTATCGCGGCTATACGGGCG) was designed to poorly target the E.coli DNA polymerase and extensively screened to ensure that it does not affect human nor viral transcripts. Use of shDaxx and shATRX was previously described elsewhere [30,31]. shZEB1.1 was obtained from the TRC library (Sigma, Inc), with targeting sequence CGCAACAATACAAGAGGTTAAACTCGAGTTTAACCTCTTGTATTGTGTC. Mutu I cells were infected with lentiviruses carrying pLKO.1-puro vector by spin-infection at 400 g for 45 minutes at room temperature. The pellets were resuspended in fresh medium and left growing overnight. The RPMI medium was replaced each day, with 2.5 μg/ml Puromycin added for selection for lentivirus transduced cells. The cells were collected after 9 days of puromycin selection, and subject to Flow cytometry quantification of EBV viral capsid antigen positive cells, and Western blot analysis.

**Supporting Information**

**Figure S1** BNRF1 co-localizes with Daxx at PML-NBs and disperses ATRX from PML-NBs. Hep2 cells were transfected with either FLAG empty vector, WT BNRF1, or the deletion constructs d26 (or d2 in panel D) and DID. Cells were fixed 2 days post transfection and co-stained with anti-FLAG, and either anti-Daxx (A), anti-PML (B), or ATRX (C) are analyzed.

**Figure S2** Signal intensity profiles analysis of BNRF1 co-localization with PML nuclear bodies. Color channel merged panels from Fig. 4 were subject to line scan signal intensity analysis as [64]. Signal intensity plots of red and green channels were plotted bellow each photo, where the x-axis runs from left to right along the yellow line drawn across several nuclear foci in each photo. Overlaps of BNRF1 signals with Daxx (A), PML (B), and ATRX (C) are analyzed.

**Figure S3** Time-course study of the effects of BNRF1 on PML and Daxx protein stability. Hep2 cells were transfected with vector control, WT BNRF1, or BNRF1-d26 mutant expression vectors. Total cell lysates were analysed by Western blot at 0, 12, 24, or 48 hrs post-transfection. Western blots were probed with antibodies to PML, Daxx, FLAG (BNRF1), or Actin, as indicated to the right.

**Table S1** Percentage of BNRF1 foci overlap with Daxx or PML foci, as observed by IF microscopy.

**Table S2** Primers used in site-directed mutagenesis of BNRF1 deletion constructs.

**Table S3** Primers used for real time detection of viral gene expression.

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**Author Contributions**

Conceived and designed the experiments: KT NT PML. Performed the experiments: KT NT JAW. Analyzed the data: KT PML. Contributed reagents/materials/analysis tools: HJD. Wrote the paper: KT PML.

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