國立臺灣大學醫學院微生物所

博士論文

Graduate Institute of Microbiology
College of Medicine
National Taiwan University

Doctoral Dissertation

BMRF1 調節 EB 病毒溶裂期基因表現及影響 BKRF3 DNA-尿嘧啶糖苷酶功能之探討

BMRF1-Mediated Regulation of Viral Gene Expression and the Biological Function of BKRF3 Uracil-DNA Glycosylase in Epstein-Barr Virus Lytic Replication

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中華民國 104 年 6 月

June, 2015

國立臺灣大學博士學位論文
口試委員會審定書

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本論文係 蘇美慈 君（學號 D97445010 ）在國立臺灣大學微生物學所完成之博士學位論文，於民國 104 年 6 月 17 日承下列考試委員審查通過及口試及格，特此證明

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誌謝

「人生有夢，築夢踏實。」築夢與逐夢的漫漫研究長路中偶有歡喜偶有傷悲，旅途中大家對我的指導與鼓勵讓我能一心一意的往夢想國度行走。謝謝陳美如老師細心的教導我身為研究人應有的邏輯思考、英文表達及研究態度，更帶領我走出國際讓我更具有世界觀。謝謝蔡錦華老師對我的研究及口頭報告有精闢的建議，有嚴厲的批評讓我能檢視自己的缺點並加以改進。謝謝林素芳老師提供實驗材料讓我的研究得以進行，也對我的論文撰寫給予許多良好的建議。謝謝張麗冠老師及陳紀如老師能夠擔任我的口試委員，在每次論文進度報告時給我非常好的意見。謝謝李重霈老師在我博士研究生涯中扮演亦師亦友的角色，讓我能一一克服實驗瓶頸及生活瑣事。謝謝李財坤老師願意讓我在他實驗室 rotation。謝謝鄧述諄老師提供酵母菌相關研究材料，讓我能在病毒實驗室以酵母菌為研究模式探討病毒蛋白激酶的功能。

EBV Lab 優秀的學長姐帶領著我一路成長茁壯，感謝錦堂學長能夠細心地回答我任何問題，能被您稱讚是"古意人"真是我的榮幸。謝謝淑君學姊總在我有實驗問題時協助我克服困難。感謝志忠學長及書銘學長對 BKRF3 酵素活性的探討，以及謝謝佩雯學姊開啟 BMRF1 功能探討之相關研究，讓我能延續這兩個研究計畫。謝謝慧慧學姊提供 reporter plasmids 讓我能完成 BMRF1 基因調控功能的研究。謝謝君翰學長及雅菁學姊給予許多實驗方法的建議。謝謝洲維學長幫我分擔許多實驗室工作及教導我如何使用螢光顯微鏡。謝謝長庚大學邱亞芳學姊對製作基因剔除病毒的技術指導。博士的訓練過程中我也遇到很多善良體貼的好夥伴與學弟妹。謝謝育欣協助我完成 EB 病毒蛋白質激酶 BGLF4 在酵母菌中的功能探討。感謝伶詩熱心地與我討論科學，並指導我的英文寫作。謝謝雅葶和育菁能夠跟我一起建立基因剃除病毒製作系統。謝謝伊華建立 BKRF3STOP 重組 EB 病毒，讓我順利進行後續的相關研究。謝謝祝元小幫手幫忙建立 EB 病毒核酸 Q-PCR 定量方法。謝謝雅琪、奐勻、培倫、庭豪、佩芳、旻潔、巧潔、姿巡、浩雲、韶文、冠廷、劉怡、宇馨、柏廷、詩婷、嘉琳、昱禎、宗諭、維筑、羅敏、冠婷、昱真、佩頤、坤詣、宜芳、楷敏、薺萱、詒捷、亭瑋、櫂杭和含修等眾多學弟妹們陪伴我走過這段逐夢之旅，讓我的博士班生活更添色彩。

人在江湖上行走，出外就是要靠朋友。再次感謝あらし小分隊的成員育欣、伶詩及祝元，在嵐精神導師的陪伴下我們共同成長茁壯，認識你們真好。謝謝摯友雅旻在我剛到台北求學時熱心地提供住處讓我棲身。謝謝美儀、玠樺、盈敏、彥秀及佳芷帶我熟悉微生物所的環境以及到處吃吃喝喝不忘帶上我，更帶我進入瑜珈的世界讓我能運動保健康。特別感謝張耀文先生能夠覺得我是塊唸書的料，願意投資我攻讀博士，讓我們能一起成長。

電話那頭，「最近過得怎樣，身體要顧好喔!」，父母親傳來暖暖的愛與關懷，兄嫂及姪子(女)們的加油打氣聲縈繞在我腦海，家族的愛讓我無後顧之憂的向夢想前進，謝謝你們的支持與鼓勵。夢想永無止境，我會帶著你們的愛繼續向前行!

中文摘要

EB 病毒是普遍感染全球人口的 gamma 型疱疹病毒。EB 病毒與 Burkitt's 淋巴瘤、Hodgkin's 症、鼻咽癌等許多人類惡性疾病形成有高度相關性。EB 病毒感染後，環狀病毒基因體利用細胞內 DNA 複製機組進行複製並潛伏在宿主細胞中。EB 病毒由潛伏再活化後，轉活化因子 Zta 及 Rta 的表現會活化一連串溶裂期基因表現以利病毒複製。EB 病毒與哺乳細胞之 DNA 複製複合體類似，包含導引酶(primase, BSLF1)、導引酶輔助蛋白(primase accessory protein, BBLF2/BBLF3)、解螺旋酶(helicase, BBLF4)、DNA 聚合酶(DNA polymerase, BALF5)、單股 DNA 結合蛋白質(single-stranded DNA-binding protein, BALF2)、尿嘧啶醣苷酶(uracil-DNA glycosylase, BKRF3)和 DNA 聚合酶輔助因子(DNA polymerase processivity factor, BMRF1)已被證實會聚集在病毒 DNA 複製區。BMRF1 除了幫助病毒 DNA 複製，同時也可作為轉活化因子調節溶裂期病毒基因體複製起始區的 BHLF1 啟動子，或作為共同活化因子調節單股 DNA 結合蛋白 BALF2 啟動子，顯示 BMRF1 在病毒溶裂期 DNA 複製及基因表現都扮演重要角色。本論文中對於 BMRF1 相關生物功能進行兩個研究主題: **(I) BMRF1 調控 BKRF3 進入細胞核，以及 BKRF3 在溶裂期病毒核酸複製複合體中所扮演的生物功能。**研究結果發現 BMRF1 可與 BKRF3 交互作用進而促使 BKRF3 由細胞質進入細胞核中，而 BKRF3 與 DNA 複製相關蛋白的結合可以提高 BKRF3 之酵素活性。剔除 BKRF3 基因導致病毒 DNA 複製功能受損，並且以互補試驗證明 BKRF3 的 leucine loop 對 BKRF3 協助病毒基因體複製是重要的。**(II)利用基因剃除病毒系統探討 BMRF1 對病毒基因的轉錄調控。**實驗結果發現 BMRF1 參與部分病毒晚期基因之轉錄調控，而 BMRF1 轉活化功能區會影響其調控病毒基因表現的功能。以免疫沉澱法搭配質譜科學分析法(Immunoprecipitation-mass spectrometry)分析與 BMRF1 相互作用之蛋白質，發現 BMRF1 不僅與 DNA 複製/修復及 RNA 剪接相關蛋白形成蛋白質複合體，也與染色質調控分子像是 BRG1 (BRM/SWI2-related gene 1)有相互作用。進一步發現

BMRF1 與 BRG1 在細胞內及體外試驗皆有交互作用。以干擾 RNA 抑制 BRG1 表現會降低 BMRF1 對部分病毒啟動子的轉活化功能，顯示 BRG1 可能參與 BMRF1 調節的病毒基因之轉錄調控。本論文證明 BMRF1 調控 BKRF3 參與細胞核內複製複合體是病毒 DNA 複製的關鍵；除了參與病毒基因體複製，BMRF1 也可能與染色質調控分子 BRG1 共同調控病毒基因表現以利 EB 病毒溶裂期複製。

關鍵字：EB 病毒，DNA 聚合酶輔助因子 BMRF1，尿嘧啶醣苷酶 BKRF3，病毒核酸複製，病毒基因表現

Abstract

Epstein-Barr virus (EBV) is a ubiquitous gammaherpesvirus that infects most of the population worldwide. EBV is highly associated with various human malignancies, including Burkitt's lymphoma (BL), Hodgkin's disease (HD) and nasopharyngeal carcinoma (NPC). After primary infection, the circularized EBV genome persists latently in the host cell, using cellular DNA replication machinery for replication. Once EBV reactivation from latency, the expression of transactivators Zta and Rta turn on cascade expression of viral lytic genes which are required for efficient viral replication. Similar to mammalian DNA replication machinery, a large protein complex including primase (BSLF1), primase accessory protein (BBLF2/3), helicase (BBLF4), DNA polymerase (BALF5), single-stranded DNA-binding protein (BALF2), uracil-DNA glycosylase (UDG, BKRF3), and DNA polymerase processivity factor (BMRF1) are demonstrated to associate with viral DNA replication compartment. Other than the role in DNA replication, BMRF1 also functions as a transactivator on BHLF1 promoter and as a coactivator on BALF2 promoter, indicating that BMRF1 plays important roles in viral DNA replication and viral gene expression. To explore the biological functions of BMRF1, two specific aims are addressed in this study. (I) The first part is to explore whether BMRF1 mediates nuclear translocation of BKRF3 and to study the biological function of BKRF3 in viral DNA replication complex during lytic cycle. It was found that BMRF1 interacts with BKRF3 and regulates the nuclear targeting of BKRF3. The interaction of BKRF3 with viral proteins in replication compartment enhances the enzymatic activity of BKRF3 during lytic cycle. In BKRF3 knockout recombinant virus, lytic DNA replication is blocked and can be rescued by *trans*-complementation of BKRF3, indicating that BKRF3 is required for EBV DNA replication and the leucine loop of BKRF3 is critical for its function. (II) The second part is to investigate the

transcription regulatory function of BMRF1 using specific gene knockout bacmid system. In gene regulation analyses, we found that BMRF1 regulates a number of viral late gene expression through its transactivation domain. According to immunoprecipitation-mass spectrometry analysis, BMRF1 associates with not only DNA replication/repair and RNA splicing association factors but also chromatin modifiers such as BRG1 (BRM/SWI2-related gene 1). Moreover, BMRF1 interacts with BRG1 in the cells and *in vitro*. Knockdown of BRG1 reduces transactivation activity of BMRF1 on a subset of viral promoters, suggesting BRG1 may participate in the regulation of BMRF1 on viral gene expression. In conclusion, BMRF1 recruits viral UDG BKRF3 to the replication compartment, which is crucial for viral DNA replication. In addition to the role in DNA replication machinery, BMRF1 may also mediate gene expression through interacting with chromatin modifier BRG1 to benefit EBV lytic replication.

Key words: Epstein-Barr virus, DNA polymerase processivity factor BMRF1, uracil-DNA glycosylase BKRF3, viral DNA replication, viral gene expression

Contents

Chapter 1: Epstein-Barr virus DNA replication and gene regulation

1.1. Discovery and characterization of Epstein-Barr Virus

Epstein-Barr virus (EBV), a member of gammaherpesvirus, is one of human DNA viruses. EBV infection occurs worldwide, and more than 95% people become infected with EBV during childhood. Most of EBV infection usually is asymptomatic or is indistinguishable from the other mild illnesses. In primary infection, EBV establishes a transient replication in the epithelium of oropharynx, and then the virus spreads throughout the lymphoid tissues to establish latent infection in B cells of the infected host. However EBV primary infection in young adulthood can cause infectious mononucleosis also known as kissing disease. Additionally, EBV infection also associates with particular forms of malignancies, such as nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma, and Burkitt's lymphoma (Longnecker et al. 2013).

The double-stranded DNA genome of EBV is approximately 172 kb in length. The B95-8 strain of EBV is the first herpesvirus be completely cloned and sequenced (accession number V01555). The B95.8 EBV containing about 85 open reading frames (ORFs) is commonly used to characterize the biological function of EBV genes (Baer et al. 1984). An 11.8 kb sequence missing from the B95-8 genome was isolated from Raji cells (Raab-Traub et al. 1980). The sequence analysis of the Raji EBV genome revealed the presence of additional leftward ORFs that refer as LF1, LF2 and LF3 (Parker et al. 1990). Reconstruction of Raji and B95-8 sequences data provides an updated wild-type EBV genome sequence (accession number AJ507799). According to the B95-8 genome

that was sequenced from an EBV DNA BamHI fragment cloned library, ORFs are defined to specific BamHI fragments which are based on fragment size, relative position and orientation within EBV genome, e.g., ORF BMRF1 for BamHI-M fragment rightward frame 1.

1.2. Latent infection of EBV

EBV DNA persists as a circular episomal form in the latently infected cells. EBV encodes for approximately 85 ORFs which are required for its life cycle. In addition to protein expression, the EBV genome encodes two classes of non-coding RNAs, small non-polyadenylated mRNAs (EBERs) and BamHI-A rightward transcripts (BARTs) (Glickman et al. 1988, Smith et al. 2000, de Jesus et al. 2003). Only a small number of genes are expressed in latently infected cells. According to the expression file of latent genes, EBV latency is categorized into four types, type 0, I, II and III. There is only EBER-1 expressed in type 0 latency. In type I program, EBV expresses EBERs, BARTs and EBNA-1 in infected cells as well as Burkitt lymphoma cells. Type II latency presented in Hodgkin's lymphoma or nasopharyngeal carcinoma cell expresses EBERs, BARTs, EBNA-1 and two latent membrane proteins (LMP1 and LMP2). During type III latency, almost all the latent proteins (EBNA-1 to -6, LMP1 and LMP2) as well as EBERs and BART2 are detected in AIDS-associated immunoblastic lymphomas and post-transplant lymphoproliferative disease (PTLD) (Klein et al. 2007).

1.3. Reactivation and lytic replication of EBV

1.3.1. Activation of lytic cycle

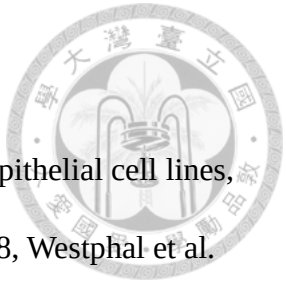
In cell culture system, EBV reactivation can be triggered by either chemical or biological agents, including TPA (12-O-tetradecanoylphorbol-13-acetate), HDAC (histone deacetylase) inhibitors, calcium ionophores, DNA demethylating agents, anti-Ig, TGF-β or hypoxia (zur Hausen et al. 1979, Datta et al. 1980, Faggioni et al. 1986, Takada and Ono 1989, Chang and Liu 2000, Jiang et al. 2006, Iempridee et al. 2011). The cascade of the EBV lytic cycle is divided into three classes of regulated lytic gene expression: immediate early (IE), early (E) and late (L) (Murata 2014). The IE gene products, Zta and Rta, are transactivators that trigger the expression of early genes. Early genes encode for replication association products that are required for viral DNA replication. Subsequently, transcription of most late viral genes is turned on following viral DNA replication. During the late phase of the lytic cycle, viral structural proteins encoded by late genes are assembled into procapsids to allow viral genomes encapsidated for the release of infectious virions. The lytic replication in normal hosts has only been detected in oropharyngeal epithelial cells (Steven et al. 1997, Pegtel et al. 2004) and in the tonsillar plasma cells (Laichalk and Thorley-Lawson 2005). Notably, the infectious viral particles have been detected in the plasma of immunosuppressed patients (Cohen 2000, Feng et al. 2004). In addition, anti-viral drugs acyclovir and ganciclovir inhibit lytic cycle of EBV and decrease the risk of post-transplant lymphoproliferative disease (PTLD) (Funch et al. 2005). Therefore, lytic replication may play a role in pathogenesis of EBV associated diseases.

1.3.2. Immediate early proteins

BZLF1 and BRLF1 have been defined as immediate early gene from latency to lytic replication (Biggin et al. 1987, Shimizu et al. 1989, Flemington et al. 1991). By induction of the lytic cycle, these two genes are expressed immediately to encode the transactivator proteins known as Zta (BZLF1, ZEBRA, EB1) and Rta (BRLF1) (Lieberman and Berk 1990, Feederle et al. 2000).

1.3.2.1. Zta

Zta, a member of the basic-zipper family of transcription factors, binds as a homodimer to AP-1 motifs and Zta response elements (ZREs) within IE and E lytic gene promoters (Farrell et al. 1989, Chang et al. 1990, Flemington and Speck 1990, Flemington and Speck 1990, Lieberman and Berk 1990). During initiation of lytic reactivation, Zta activates the Rta promoter (Rp), and activates many early lytic genes by the synergistic regulation with Rta (Adamson and Kenney 1998, Feederle et al. 2000, Bhende et al. 2005). Zta also plays an important role in lytic DNA replication, binding to series of ZREs in the lytic replication origin (oriLyt) (Hammerschmidt and Sugden 1988, Schepers et al. 1993, Fixman et al. 1995). Mutation of Zta residues, including S173, C189, Y180 and K188, causes a defect in lytic DNA replication rather than lytic gene transcription (Heston et al. 2006, El-Guindy et al. 2007). Zta interacts with other core viral replication proteins to promote formation of the initial replication complex (Takagi et al. 1991, Zhang et al. 1996, Gao et al. 1998).



1.3.2.2. Rta

In some of latently infected cell lines, especially EBV infected epithelial cell lines, expression of Rta induces lytic cycle progression (Ragoczy et al. 1998, Westphal et al. 1999, Feederle et al. 2000). The Rta-deleted mutant EBV bacmid is incompetent to enter the lytic infection or to express Zta efficiently, indicating that Rta is an essential activator of Zp promoter in epithelial cells (Feederle et al. 2000). Similar to Zta, Rta directly binds to the oriLyt of EBV and functions as an essential protein for lytic replication (Hammerschmidt and Sugden 1988, Gruffat and Sergeant 1994, El-Guindy et al. 2013). Rta activates lytic promoters through multiple pathways. In some cases, Rta directly binds to a Rta response element ($GGCCN_9GTGGTG$, RRE) to activate a number of lytic genes (Kenney et al. 1989, Gruffat et al. 1992, Quinlivan et al. 1993, Gruffat and Sergeant 1994).

On the other hand, Rta activates lytic gene expression through indirect mechanisms. First, Rta stimulates Rp through Rp SP1 motifs (Ragoczy and Miller 2001, Liu and Speck 2003). The regulatory complex containing Rta, SP1 and MCAF1 binds to Sp1-binding sites (Chang et al. 2005). Second, Rta increases the phosphorylation level of p38 and c-Jun N-terminal kinases to activate ATF2 transcription factors which bind to and activate Zp (Adamson et al. 2000). Third, Rta also interacts with the histone acetylase CBP (CREB-binding protein) in Raji cells. The interaction between BRLF1 and CBP is important for Rta-mediated activation of the early lytic EBV gene SM. (Swenson et al. 2001). Finally, the interaction between Rta and the tumor susceptibility gene 101 (TSG101) is critical for the transactivation ability of Rta to activate some viral late genes (Chua et al. 2007).

1.3.3. Early gene products

Early lytic gene products have a wide range of functions, such as DNA replication, metabolism, and blockade of antigen processing (Odumade et al. 2011). According to protein interaction networks, a set of early proteins compose the replication complex to benefit viral lytic replication.

1.3.3.1. Lytic DNA replication

Lytic EBV DNA replication initiates at the oriLyt origin. Two copies of oriLyt, $oriLyt_L$ and $oriLyt_R$, are present in the EBV genome. Six core replication proteins are absolutely required to coordinate with Zta for the replication of plasmids containing oriLyt (Fixman et al. 1992, Fixman et al. 1995). These proteins are the DNA polymerase BALF5, single-stranded DNA-binding protein BALF2, DNA polymerase processivity factor BMRF1, helicase BBLF4, primase BSLF1 and primase accessory protein BBLF2/3 (Table 1). This complex opens the DNA helix at the oriLyt and synthesizes RNA primers allowing DNA replication to proceed by BALF5 (Fujii et al. 2000). In 2013, El-Guindy et al. demonstrated that Rta plays an essential role in the process of lytic DNA replication (El-Guindy et al. 2013). In addition to viral replication proteins, cellular proteins also participate in lytic DNA replication. Cellular transcription factors SP1 and ZBP89 bind to the oriLyt and interact with the BALF5/BMRF1 complex (Baumann et al. 1999). Moreover, BBLF2/3 provides tethering point on oriLyt for the EBV replication complex through interaction with the zinc finger DNA binding protein ZBRK1 and the KAP-1 corepressor, suggesting cellular transcription factors tether the viral replication proteins to oriLyt (Liao et al. 2005).

1.3.3.2. DNA polymerase processivity factor, BMRF1

BMRF1 is the major early phosphoprotein induced during EBV infection (Cho et al. 1985, Li et al. 1987). During lytic EBV replication, BMRF1 co-localizes with BALF5 at intranuclear replication compartments (Kiehl and Dorsky 1991). The nuclear localization signal (NLS) of BMRF1 has been defined at the carboxyl-terminal domain (amino acids 383 to 397) (Zhang et al. 1999) and Fig. 1). The crystal structure of C-terminus-truncated BMRF1, forms head-to-head homodimer or tetrameric ring in solution, shows similarity with other processivity factors, such as herpes simplex virus UL42, cytomegalovirus UL44 and human proliferating cell nuclear antigen (PCNA) (Murayama et al. 2009). BMRF1 associates with BALF5/Hsp90 complex to promote nuclear transport of BALF5 (Kawashima et al. 2013). In addition to DNA polymerase accessory function, BMRF1 can transcriptionally activate the BHLF1 promoter through SP1 and ZBP-89 binding motif (Zhang et al. 1996). BMRF1 possesses a transactivation domain at amino acid 379 to 388 (Zhang et al. 1999 and Fig. 1). In addition, BMRF1 functions as a coactivator which interacts with Zta to enhance Zta-mediated transcription of the BALF2 promoter through ZRE (Nakayama et al. 2009). Besides regulation of viral gene promoters, BMRF1 activates transcription of the cellular gastrin gene which mediated by two SP1/ZBP-89 binding sites in the gastrin promoter (Holley-Guthrie et al. 2005). Therefore, BMRF1 plays dual biological functions on DNA replication and gene transcription.

The BMRF1 gene product has been purified and characterized as phosphorylated forms of 52 and 50 kDa and an unphosphorylated form of 48 kDa (Cho et al. 1985). The phosphorylation of BMRF1 is mediated by viral protein kinase BGLF4 (Chen et al.

2000, Gershburg and Pagano 2002). BGLF4 induces hyperphosphorylation of BMRF1 at four residues Ser-337, Thr-344, Ser-349 and Thr-355 (Yang et al. 2008). Notably, BGLF4 represses BMRF1 transactivation on the BHLF1 promoter, suggesting the BGLF4-mediated phosphorylation may modulate the biological function of BMRF1 (Yang et al. 2008).

1.3.3.3. Uracil-DNA glycosylase, BKRF3

In addition to viral DNA replication core proteins, BKRF3, the viral homolog of uracil-DNA glycosylase (UDG), is shown to enhance oriLyt-dependent DNA replication about 2-folds in a cotransfection-replication assay (Fixman et al. 1995). BKRF3 excises uracil in both dsDNA and ssDNA but has a preference for ssDNA *in vitro* (Lu et al. 2007). Expression of BKRF3 is regulated mainly by Rta. The efficiency of EBV lytic DNA replication is slightly affected by BKRF3 siRNA, whereas cellular uracil-DNA glycosylase UNG2 siRNA or inhibition of both cellular and viral UNG activities by Ugi represses EBV lytic DNA replication (Lu et al. 2007). As demonstrated in the gene knockdown experiment, BKRF3 as well as the cellular UNG2 is involved in viral DNA replication and/or repair to ensure the replication fidelity of viral DNA (Lu et al. 2007). According to the crystal structure analysis, BKRF3 shares considerable similarity in the overall structure with proteins in the family 1 UDGs which are active against uracils in ssDNA and dsDNA (Geoui et al. 2007 and Fig. 2). Four out of the five catalytic motifs are conserved completely, whereas the fifth domain carries a seven-residue insertion in the leucine loop, hinting that the leucine loop of BKRF3 may play a role in viral replication.

1.3.4. Late gene products

EBV encodes a number of glycoproteins and structure proteins during late phase of lytic cycle. The glycoproteins present in the classical membrane antigen (MA) complex are gp350/220 (BLLF1) and gH (gp85, BXLF2) (Edson and Thorley-Lawson 1981). The BLLF1 ORF is transcribed as two mRNA transcripts. The smaller of BLLF1 transcripts is generated by an in-frame splicing (Biggin et al. 1984). The gp350/220 mediates binding of EBV to CD21 receptor on the B-cell as known as CR2 (Fingeroth et al. 1984, Nemerow et al. 1989). On the other hand, the glycoprotein complex gH/gL/gp42 contributes to viral-cell fusion. The glycoprotein gH (BXLF2) forms a heterotrimeric complex with two more glycoproteins, gL (BKRF2) and gp42 (BZLF2). The gp42 binds to HLA class II followed by gH/gL complex mediated virus-cell membrane fusion (Hutt-Fletcher and Lake 2001).

BALF4 (gB), as known as gp110, is also involved in virus-cell membrane fusion. Higher levels of gB are observed in the nuclear membrane of cells producing B95-8 EBV than in mature enveloped virions (Gong et al. 1987, Gong and Kieff 1990, Papworth et al. 1997). A gp110 mutant EBV was not released from the cell (Herrold et al. 1996). Virions containing gB increase EBV infectivity and a wider cell tropism. In addition, the gB can mediate membrane fusion in a virus-free assay (Haan et al. 2001). A recent study reported that gB directly interacts with neuropilin 1 (NRP1) to promote EBV infection of nasopharyngeal epithelial cells through activating NRP1-dependent receptor tyrosine kinase (RTK) signaling (Wang et al. 2015). Additionally, EBV also encodes four membrane proteins: gp60 (BILF1), gp78 (BILF2), gp150 (BDLF3) and BMRF2 (Modrow et al. 1992, Nolan and Morgan 1995, Johannsen et al. 2004). EBV

lacking gp150 infects B cells normally but has an enhanced infection rate for epithelial cells (Borza and Hutt-Fletcher 1998). Transmembrane glycoprotein BMRF2 binds to cellular integrins α5β1 at the basal membrane of polarized oropharyngeal epithelial cells (Xiao et al. 2007). BMRF2 interacts with BDLF2, a component of the EBV virion tegument, to alter the epithelial cell morphology through reorganization of the actin cytoskeleton (Loesing et al. 2009).

In addition to glycoproteins, a number of virion capsid antigens have been characterized as products of late genes. The major capsid protein p160 (BcLF1) and three small capsid proteins, p18, p23 and p40 (BFRF3, BLRF2 and BdRF1) have been identified (van Grunsven et al. 1993, van Grunsven et al. 1993, Reischl et al. 1996).

1.4. EBV gene expression and regulation

1.4.1. Regulation of early lytic gene

In EBV genome, transcriptional activation of many early promoters, such as that of BMRF1, requires both Zta and Rta activation (Cox et al. 1990, Feederle et al. 2000). In contrast, the early SM promoter, which contains Zta and Rta response elements, can be activated by Rta expression alone even in the context of the intact viral genome in Raji cells. (Ragoczy and Miller 1999, Swenson et al. 2001). In addition, the divergent two early promoters within the oriLyt of EBV, BHLF1 and BHRF1, have both ZREs and RREs. By reporter assays, the BHRF 1 promoter is more responsive to Rta than the BHLF1 promoter (Hardwick et al. 1988, Cox et al. 1990). Both the BHLF1 and BHRF1 promoters are activated by Zta alone (Hardwick et al. 1988). The GC-rich transcript of

BHLF1 annealed to its DNA template during early stage of lytic replication. The RNA-DNA hybrid is crucial for initial strand separation and loading of core replication proteins, indicating the transcriptional regulation of oriLyt plays an important role during lytic reactivation (Rennekamp and Lieberman 2011).

1.4.1.1. Regulation of host transcription factors on viral early gene expression

The transcription factor SP1 is a zinc finger transcription factor that binds to GC-rich motifs of promoters. During lytic reactivation, SP1 coordinates with p53 to regulate gene expression of Zta through binding to the ZID element of Zta promoter (Chua et al. 2012). In addition, SP1 also regulates the expression of Rta via interaction with MCAF1 and Rta (Chang et al. 2005). Moreover, BMRF1 alone can activate the BHLF1 and the gastrin promoters without expression of BZLF1, suggesting that BMRF1 might enhance SP1-mediated transcriptional activation through interacting with SP1 bound to GC-rich motifs (Zhang et al. 1996, Zhang et al. 1997, Holley-Guthrie et al. 2005).

1.4.1.2. BGLF4-mediated regulatory effects on the transactivation activities of Zta and BMRF1

EBV BGLF4, a Ser/Thr protein kinase, functions as a CDK-like protein kinase by phosphorylating several viral proteins including BMRF1, viral nuclear antigen leader protein EBNA-LP, viral transactivatior EBNA-1 and -2 and Zta etc. (Chen et al. 2000, Gershburg and Pagano 2002, Zhu et al. 2009). BGLF4 also phosphorylates cellular proteins as well as Cdks, including condensin complex and TOP2α etc. (Lee et al. 2007,

Lee et al. 2008, Zhu et al. 2009). In reporter assays, co-expression of BGLF4 inhibits BMRF1 transactivation activity on BHLF1 promoter. Interestingly, BGLF4 enhances the transactivation activity of Zta and the synergistic activation of BMRF1 and Zta on the BHLF1 promoter (Yang et al. 2008). It suggests that BGLF4-mediated phosphorylation of BMRF1 or Zta may regulate its transcriptional functions.

1.4.2. Regulation of late viral genes

Late genes are traditionally defined as genes expressed after viral lytic DNA replication. Late gene expression is repressed by treatment with inhibitors of the viral DNA polymerase, such as phosphonoacetic acid (PAA) (Summers and Klein 1976, Rickinson and Epstein 1978), phosphonoformic acid (Datta and Hood 1981), and acyclovir (Colby et al. 1980). In addition, the Raji cell line which contains EBV with a deletion in single-strand DNA-binding protein BALF2 is failure in lytic viral DNA replication and late gene expression (Decaussin et al. 1995). Those data suggest that the activity of BALF5 and lytic replication of the viral DNA are required for late gene expression. Some of late promoters can be activated in athe DNA replication independent manner by reporter assay, including BcLF1, BFRF3 and BLLF1 (Serio et al. 1997, Lu et al. 2006). Several studies reported that viral replication is not required for activation of late promoters. In 293 cells, expression of Rta induces a subset of late gene expression in Zta-deleted virus with a defective viral replication (Feederle et al. 2000). In Raji cells, Rta also activates some late genes in the absence of viral replication (Ragoczy and Miller 1999, Lu et al. 2006).

1.5. Interaction of viral transcription factors and cellular factors in lytic phase

During lytic infection, Zta directly interacts with histone modification complexes, including CBP and p300, resulting in histone acetylation for transcription (Adamson and Kenney 1999, Zerby et al. 1999, Chen et al. 2001, Deng et al. 2003). Zta also interacts with a number of basic transcription factors, including TFIID and TFIIA (Lieberman and Berk 1991, Chi and Carey 1993, Lieberman and Berk 1994, Chi et al. 1995, Lieberman et al. 1997). Moreover, the nuclear protein 53BP1, a component of the ATM DNA damage response pathway, interacts with Zta and may involve in the viral lytic cycle (Bailey et al. 2009). In addition to interaction with SP1, Rta directly interacts with the cellular Pit-Oct-Unc (POU) domain transcription factor Oct-1 to activate its own promoters (Robinson et al. 2011).The recruitment of CBP, TRAP/Mediator and SWI/SNF complexes mediated by Rta provides well control of viral gene expression during Kaposi's sarcoma-associated herpesvirus (KSHV) lytic reactivation (Gwack et al. 2003). Therefore, the interactions between viral transactivator and cellular transcription factors and histone or chromatin modifiers support the efficiency of viral lytic replication.

1.6. Aims of this study

In previous studies, BMRF1 has been proposed to function as a transactivator or a coactivator to mediate viral early promoters of BHLF1 and BALF2. It is possible that BMRF1 also plays a crucial role in the expression of other EBV genes. To explore the transcription regulatory function of BMRF1 in the context of the whole viral genome, we generated a BMRF1 knockout EBV bacmid and screened with an EBV DNA microarray to identify the BMRF1-regulated genes of EBV. In addition, we are interested in cellular factors which participate in the BMRF1-mediated gene activation. For this purpose, immunoprecipitation-mass spectrometry analysis was performed to identify the BMRF1-interacting proteins, and clarify the biological functions of the interaction of BMRF1-cell factors. On the other hand, our previous study suggests that both cellular UNG and viral UDG BKRF3 contribute to viral DNA replication during lytic cycle in gene knockdown experiment. However, the mechanism of BKRF3 nuclear targeting is still unknown. As the result of the expression of BKRF3 was not completely depleted by siBKRF3, it is difficult to conclude whether BKRF3 is crucial for lytic DNA replication. Therefore, we established the BKRF3 knockout EBV bacmid to clarify the genuine biological function of BKRF3.

Chapter 2: Uracil-DNA glycosylase BKRF3 contributes to Epstein-Barr virus DNA replication through physical interactions with proteins in viral DNA replication complex

2.1. Introduction

During EBV lytic infection, the core viral DNA replication complex contains eight virus-encoded proteins, including BZLF1 (oriLyt-binding protein), BRLF1 (immediate early transactivator), BALF5 (DNA polymerase), BMRF1 (also called EA-D, polymerase processivity factor), BALF2 (single-stranded DNA-binding protein), BBLF4 (helicase), BSLF1 (primase) and BBLF2/3 (primase accessory protein) (Fixman et al. 1995, El-Guindy et al. 2013). In addition, EBV encodes a viral uracil-DNA glycosylase (UDG), BKRF3, which is a homolog of the human uracil-*N*-glycosylase (UNG) family (Lu et al. 2007).

The UDG superfamily comprises five protein families which share a similar structural organization but lack sequence homology at their active sites (Pearl 2000, Mi et al. 2009). Among them, the UNG family (family 1) is the most ubiquitous and conserved across species (Yonekura et al. 2009). Mammalian UNG2, one of the two isoforms encoded by the *UNG* gene through alternative splicing (Nilsen et al. 1997), is the major UNG localized in the nucleus (Slupphaug et al. 1995). The other isoform is the mitochondrion-specific UNG1. These two isoforms are encoded by the same gene and differ in the first 35 and 44 residues on their N termini but are identical in the remaining 269 residues (Nilsen et al. 1997). Under normal physiological conditions,

uracils are possibly introduced into DNA by two major processes, including misincorporation of dUMP and spontaneous deamination of cytosine within DNA. The replicative incorporation of dUMP generates a U·A base pair (Gadsden et al. 1993). Alternatively, deamination of cytosine yields a G·U mismatch, and leads to G·C-to-A·T transition if the lesion is not repaired before next round replication. Normally, T-to-U or C-to-U nucleotide changes are corrected by one of the two base excision repair (BER) pathways, namely, the short- and long-patch pathways, which are initiated following recognition of uracil by UNG (Lindahl 1974). The N-glycosylic bond between uracil and deoxyribose is then hydrolyzed by UNG, creating an apurinic/apyrimidinic (AP) site (Krokan et al. 2002, Barnes and Lindahl 2004). The 5'end of the AP site is cleaved by AP endonuclease, and the resulting single-strand break can be subsequently processed via either a short-patch or a long-patch repair pathway (Pascucci et al. 1999, Scharer and Jiricny 2001).

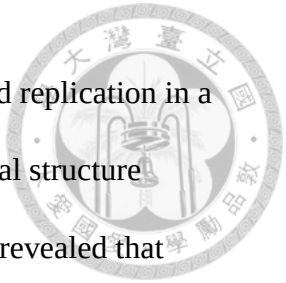
To operate together with the DNA replication machinery, different UNG molecules use various strategies to translocate into the nucleus. For example, human UNG2 is transported to the nucleus by an unusual nuclear localization signal (NLS) in the N terminus (Otterlei et al. 1998) and recruited to replication foci, through the physical interactions of its N-terminal non-catalytic domains with PCNA and replication protein A (RPA) to benefit DNA replication (Ko and Bennett 2005). UNG2 expression is highly regulated by the cell cycle, maximum levels and enzyme activities being detectable during late G_1 to early S phase (Haug et al. 1998). The cellular turnover, association with RPA and modulation of catalytic activity of UNG2 are regulated through distinct CDK-mediated phosphorylation (Hagen et al. 2008). The interactions of UNG2 with

PCNA and RPA contribute to efficient postreplicative repair of misincorporated uracils in newly synthesized DNA (Otterlei et al. 1999). In addition, UNG2 also function in pre-replicative repair of U:G mismatch through direct interaction with DNA repair protein XRCC1 (Akbari et al. 2010). Previously, it was found that overexpression of human UNG2 causes cell cycle delay and increases DNA damages in fission yeast, suggesting uncoordinated UNG2 activity may induce potential DNA damages (Elder et al. 2003). Thus, specific interactions with various DNA replication or repair proteins may provide a sophisticate regulation of UDG function.

During herpesvirus infections, various cellular components of the DNA repair machineries also participate in viral replication compartments to either stimulate or inhibit viral DNA replication. Both non-homologous end joining (NHEJ) and homologous recombination repair (HRR) and chromatin remodeling factors accumulate in HSV-1 replication compartments (Taylor and Knipe 2004). Mismatch repair (MMR) and HRR factors were found co-localized within EBV replication compartments (Daikoku et al. 2006, Kudoh et al. 2009). Additionally, it was suggested that the modulation of cellular BER pathway may play an important role in HCMV replication (Ranneberg-Nilsen et al. 2006). Depletion of UNG2 with a short hairpin RNA (shRNA) approach attenuated the viral DNA replication and virion production in Kaposi's sarcoma-associated herpesvirus (KSHV)-positive cells, induced by 12-Otetradecanoylphorbol-13-acetate (TPA) and sodium butyrate (SB) for lytic replication (Verma et al. 2006). In our previous study, EBV replication was dramatically reduced in the presence of Ugi, which can block both cellular and viral UDG activities (Lu et al. 2007). These observations implicate that cellular UNG2 contributes to viral

DNA replication.

All human herpesviruses encode conserved UDGs that were found in viral DNA replication compartments. Different UDG mutant viruses were also generated to explore exact function of herpes viral UDG during replication. For example, the UDG of HSV-1, UL2, was shown to be associated with the viral DNA polymerase UL30, and required for efficient virus replication and reactivation in neural cells (Bogani et al. 2009, Bogani et al. 2010); In a mouse infection model, recombinant HSV-1 with truncative mutant of UL2 replicates less efficienly in the nervous system than the wild-type virus does (Pyles and Thompson 1994). HCMV UDG UL114 associates with viral polymerase processivity factor UL44 for targeting into nucleus, and forms complexes with viral DNA polymerase UL54 (Prichard et al. 2005, Strang and Coen 2010). The association with UL44 enhances the UDG activity of UL114 and increases the efficiency of viral DNA synthesis (Ranneberg-Nilsen et al. 2008). Deletion of HCMV UL114 in recombinant virus delayed viral replication in quiescent fibroblasts (Prichard et al. 1996, Courcelle et al. 2001). However, the Varicella-Zoster virus (VZV) UDG mutant virus, with a deletion of a.a. 160 to 282 of ORF59, grew to similar titer as the parental virus in vitro (Reddy et al. 1998). These observations suggest that viral UDGs may be equipped for viruses to replicate well in quiescent cells, where cellular UNG expression is limited. Nevertheless, individual herpesviral UDG may have unique features to promote viral DNA replication. Previously, we found EBV UDG BKRF3 co-localized with viral DNA polymerase BALF5 in immunofluorescence microscopy (Lu et al. 2007). However, the nuclear targeting mechanism of BKRF3 and the function of BKRF3 in viral replication compartment remain to be elucidated.



BKRF3 was first shown to enhance EBV oriLyt-initiated plasmid replication in a transient co-transfection replication assay (Fixman et al. 1995). Crystal structure analysis of BKRF3 in complex with the UDG inhibitor protein (Ugi) revealed that BKRF3 shares considerable similarity of overall structure with proteins in the family 1 UNGs. Four out of the five catalytic motifs are conserved completely, whereas the fifth domain carries a seven-residue insertion in the leucine loop, implicating leucine loop of BKRF3 may play a role in viral replication (Geoui et al. 2007). Previously, we characterized the biochemical properties of BKRF3 with DNA glycosylase and mutator assays. BKRF3 was able to complement the phenotype of an *E. coli ung* mutant and showed a higher efficiency in removing uracil from artificial ssDNA probe than dsDNA in vitro. Using short interfering RNA (siRNA) to knock down BKRF3 expression caused an approximately 20% decrease in viral DNA replication in EBV-positive NA cells (Lu et al. 2007). In this study, we aimed to examine further the role and functional domains of BKRF3 in EBV lytic replication. We show that BKRF3 is translocated into the nucleus and colocalizes with the replication loci when EBV-positive cells are induced into the lytic cycle. This observation is supported by the fact that BKRF3 was immunoprecipitated with other EBV proteins within the replication compartment. Using a bacmid system, we show that a BKRF3 knockout recombinant EBV is defective for lytic DNA replication, and this defect can be restored by the expression of wild-type or the catalytic domain mutant of BKRF3, but not the leucine loop mutant of BKRF3. In addition to UDG activity, the interaction of BKRF3 with viral DNA replication compartment is critical for EBV lytic DNA replication.

2.2. Results

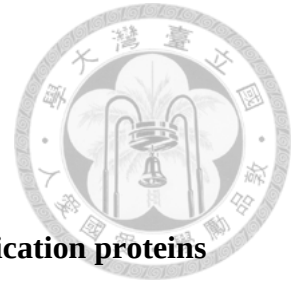
2.2.1. BKRF3 was translocated into the nucleus during EBV lytic replication

Previously, we showed that BKRF3 expression could be detected in EBV-positive NA cells upon EBV reactivation in both the nuclear and cytosolic fractions (Lu et al. 2007). However, when BKRF3 was transiently expressed alone in EBV-negative 293 cells, it was located only in the cytoplasm, suggesting that interaction of BKRF3 with other viral factors may be required for BKRF3 nuclear targeting. Here, we examined further the localization of BKRF3 with or without EBV lytic cycle induction to gain a better understanding of the expression dynamics of proteins interacting with BKRF3 in the cells. A Flag-BKRF3 expression plasmid was transfected into an Rta-inducible EBV-positive 293-derived cell line, EREV8, and the lytic cycle was induced with doxycycline at 24 h after transfection. At 48 h postinduction, protein expression was detected using an immunofluorescence assay. As shown in Fig. 3A, without Rta induction, BKRF3 was mainly distributed in the cytoplasm, whereas, with Rta induction, BKRF3 distribution moved into the nuclei, as evidenced by the superimposed BKRF3 and Hoechst-stained images. Moreover, BKRF3 showed a co-localized distribution with the EBV DNA polymerase, BALF5. In subcellular fractionation and immunoblotting analysis, most of the BKRF3 protein was detected in the cytoplasmic fraction before Rta induction by doxycycline, and shifted to the nuclear fraction after induction (Fig. 3B). Similarly, transfection of Flag-BKRF3 and Rta plasmids into an EBV-positive NPC cell line, NA, resulted in a cytoplasmic distribution of BKRF3 in the absence of Rta expression. In the presence of Rta, BKRF3 was translocated into the nuclei and colocalized with BALF5 at discrete sites (Fig. 3C), possibly the replication

compartments (Daikoku et al. 2005). More than 90% of Flag-BKRF3-expressing cells showed nuclear staining pattern and co-localized with BALF5, suggesting that BKRF3 is recruited to the nuclei through association with the viral DNA replication machinery.

To monitor the temporal expression profile of BKRF3 during the stages of EBV replication, EBV-positive NA cells were induced with TPA-SB into lytic replication. Protein expression of several lytic-cycle proteins, Rta, Zta, BMRF1, BGLF4 and BKRF3, as well as cellular UNG1 and UNG2, was determined at various time points up to 60 h postinduction. As shown in Fig. 3D, expression of BKRF3 in NA cells was detectable from 12 h postinduction and increased significantly between 24 and 36 h postinduction. After that, the expression of BKRF3 remained relatively stable through to the end of the time course. Expression of UNG1, which is one of the *UDG* products located in the mitochondria, was not affected by the expression of BKRF3 or other viral proteins. On the contrary, expression of UDG2, which is the nucleus-localized UDG, gradually decreased over the time course of induction. To determine whether this decrease of UDG2 expression caused an overall reduction of glycosylase activities, total cell lysates were collected at each time point and subjected to a DNA glycosylase assay (Lu et al. 2007). As illustrated in Fig. 3E, incubating ^{32}P-labeled 45-mer probes with cell lysates containing DNA uracil glycosylases results in AP site cleavage following alkaline treatment, generating ^{32}P-labeled 20-mer fragments. The probe cleavage rate was about 76% before induction and increased to 90% at 60 h postinduction (Fig. 3E). The time course assay indicated that, after the EBV lytic cycle was induced, total DNA glycosylase activities increased slightly even though the expression of cellular UNG2 was reduced; and the gain of total UDG activities is likely from the increased

expression of BKRF3 during EBV lytic replication.

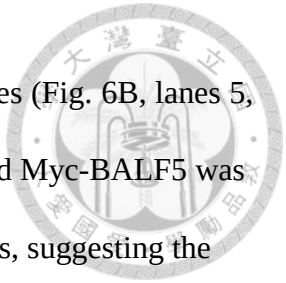


2.2.2. Physical interactions of BKRF3 with other EBV DNA replication proteins and the nuclear targeting of BKRF3

Because participation of UDG in the DNA replication core machinery was seen for both mammalian UNG2 and some viral UDGs, we performed co-immunoprecipitation assays to examine the physical association between BKRF3 and EBV replication-associated proteins. To analyze the interactions further, Myc-BALF5, Rta and Flag-BKRF3 expression plasmids were transfected into 293T cells and protein expression was detected by western blotting (Fig. 4A). In the co-immunoprecipitation reaction with anti-Flag antibody, Rta and Myc-BALF5 were detected on the immunoblot with respective antibodies, indicating a complex comprised of at least these three proteins (Fig. 4B, lane 7). Moreover, Rta or Myc-BALF5 also formed a protein complex with Flag-BKRF3 (Fig. 4B, lanes 4 and 5). Alternatively, the immunocomplexes were captured with anti-Rta antibody and immunoblotted, which indicates interaction between Rta and Myc-BALF5 (Fig. 4C, lane 6). Together, these results suggest that Flag-BKRF3, Rta and Myc-BALF5 form a complex through direct or indirect interactions. However, Flag-BKRF3 was not detected in the Rta antibody-captured immunocomplex, possibly because Rta antibody may interfere with the complex formation or the interaction among Rta, BALF5 and BKRF3 prevents epitope recognition by Rta antibody.

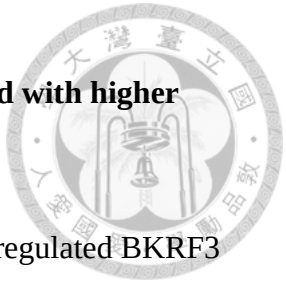
Since BKRF3-interacting proteins, Rta and BALF5, localize to replication compartments and are required for lytic EBV replication (Fixman et al. 1992, Park et al.

2008, El-Guindy et al. 2013), we examined whether Rta or BALF5 can translocate BKRF3 into nucleus. To this end, HA-BKRF3 plasmid was cotransfected with vector control or plasmid expressing Rta or Myc-BALF5 into HeLa cells and examined with immunofluorescence staining. In Rta-expressing cells, BKRF3 predominantly distributed in the cytoplasm as that in BKRF3-only cells, indicating expression of Rta did not change the cytoplasmic distribution of BKRF3 (Fig. 5A, upper panels). Interestingly, we found Myc-BALF5 displayed a partial colocalization pattern with BKRF3 in the cytoplasm (Fig. 5A, lower panel). Possible candidates that can promote the nuclear targeting of BKRF3 were searched. Previously, UL114, the UDG of HCMV, was found to associate with DNA polymerase processivity factor UL44 in HCMV-infected cells (Ranneberg-Nilsen et al. 2008). A recent study further revealed that EBV DNA polymerase processivity factor BMRF1 interacts with BALF5 through the assistance of HSP90 to promote BALF5 nuclear targeting (Kawashima et al.). Thus, we explored whether BMRF1 can directly interact with and promote the nuclear targeting of BKRF3, or the complex formation of BMRF1-HSP90-BALF5 is required for nuclear targeting of BKRF3. In immunofluorescence analysis, we found coexpression of BMRF1 can effectively promote the translocation of BKRF3 from cytoplasm into the nucleus in about 90% of coexpression cells (Fig. 5B), suggesting the interaction with BALF5 is not required for this process. In co-immunoprecipitation assay, BKRF3 was detected in the immunocomplexes captured with anti-HA antibody in HA-BMRF1- and Flag-BKRF3-coexpressed cells (Fig. 6A, lane 5). Furthermore, BKRF3 and BALF5 also were detected in BMRF1 associated immunocomplexes in the presence of all three proteins (Fig. 6A, lane 7). Reciprocally, BMRF1 and BALF5 were



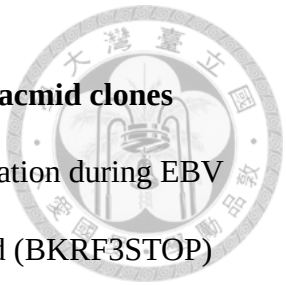
also detected in the anti-Flag antibody pulled down immunocomplexes (Fig. 6B, lanes 5, 6). Interestingly, the amount of HA-BMRF1 pulled down was less and Myc-BALF5 was not captured by anti-Flag antibody in the presence of all three proteins, suggesting the triple complex may be detected by anti-Flag antibody less efficiently (Fig. 6B, lane 7). Taken together, data here indicate that BKRF3 interacts with viral replication-associated proteins, including BALF5, Rta, and BMRF1, and the nuclear targeting of BKRF3 is regulated by BMRF1.

We then used GST-pull down assay to map different domains on BKRF3 responsible for interacting with different DNA replication proteins. GST-BKRF3 wild-type and a series of deletion mutants were generated (Fig. 7A). Indeed, BALF5, Rta or BMRF1 expressed in 293T cells was pulled-down by bacterially expressed GST tagged wild-type BKRF3 (Fig. 7B to D). GST-fused wild-type and mutant BKRF3 proteins were used in GST pull-down assays to identify the domain important for interacting with BALF5 (Fig. 7E), Rta (Fig. 7F) or BMRF1 (Fig. 7G). BALF5 and Rta were pulled-down by GST-tagged wild-type BKRF3 and BKRF3 mutants d(1-30), d(28-83) and d(81-166), but not by d(164-255). It suggests that residues 164 to 255 of BKRF3 are crucial for its interaction with Rta and BALF5. On the other hand, BMRF1 was pulled-down by the GST-tagged wild-type BKRF3 and all BKRF3 mutants except d(81-166), suggesting BKRF3 may form complexes with BMRF1 with a different interacting domain. The interacting abilities of different GST deletion clones with BALF5, Rta or BMRF1 are listed in Fig. 7A. In summary, the C-terminal region of BKRF3 is important for interaction with BALF5 and Rta, whereas middle region of BKRF3 is critical for interacting with BMRF1.



2.2.3. Complex formation of BKRF3 with viral proteins correlated with higher UDG activity of BKRF3 during EBV reactivation

As shown in Fig. 3, the reduced cellular UNG2 coupled with upregulated BKRF3 during EBV lytic cycle, implying BKRF3 may restore the UDG activity in virus-replicating cells. Because we have shown that the UDG activity of purified recombinant BKRF3 is about 10-fold less than that of *E. coli* UDG in an *in vitro* UDG assay (Lu et al. 2007), we wondered whether BKRF3 may function through interaction with proteins in the viral DNA replication complex to achieve a stronger UDG activity *in vivo*. To test this hypothesis, Flag-BKRF3 proteins were enriched from Rta or vector-transfected NA cell lysates by immunoprecipitation with anti-Flag antibodies, and immunocomplexes were subjected to UDG assays. The immunoprecipitation-Western blotting analysis indicates that BKRF3 forms a complex with BALF5, Rta and BMRF1 as described above (Fig. 8A, lane 8). Simultaneously, the Rta-transfected immunocomplex associated with BKRF3 conferred a significantly greater level of UDG activity (92% of probe cleavage), compared to that of the lysate without Rta transfection (49% of probe cleavage, Fig. 8B, lanes 7 and 8). The BKRF3 complexes associated with viral replicating proteins, including BALF5, Rta and BMRF1, indeed displayed enhanced UDG activity. Although it cannot be excluded that the enhancement may be attributed to interaction with other viral proteins, resulting in post-translational modification or enhanced stability of BKRF3, it is evident that the formation of BKRF3-associated complexes enhanced BKRF3 UDG activity during EBV reactivation.



2.2.4. Generation of a BKRF3 knockout and the revertant EBV bacmid clones

To investigate further the involvement of BKRF3 in DNA replication during EBV lytic replication, we constructed the p2089BKRF3STOP EBV bacmid (BKRF3STOP) by an allelic exchange procedure (Jia et al. 2004). A three-frame stop codon cassette was inserted at amino acid 73 of BKRF3, and the incorporation of the cassette in BKRF3 was confirmed by restriction enzyme digestion of the inserted NheI site next to the stop codons (Fig. 9A). Using BKRF3STOP bacmid as the backbone, BKRF3 revertant bacmid (K3R) was constructed by allelic exchange with wild-type BKRF3 sequence (Fig. 9B). Compared to the wild-type bacmid p2089, the 24.6-kb DNA fragment containing BKRF3 was cut into two fragments of 16.8 kb and 7.8 kb in the BKRF3STOP bacmid by NheI digestion. The stop codon cassette was replaced with wild-type sequence of BKRF3 in revertant K3R and resulted in the 24.6-kb BKRF3 containing fragment (Fig. 9B and C right panel). Furthermore, the BamHI fragmentation analysis was performed to confirm that no other recombination sites except the BKRF3 target site (Fig. 9C, left panel). Wild-type p2089, BKRF3STOP and K3R bacmids were transfected into 293TetER cells, in which Rta expression can be induced by tetracycline for the subsequent progression of the lytic cycle genes (Chen et al. 2009), to establish 293TetER/p2089 (p2089), 293TetER/p2089BKRF3STOP (BKRF3STOP) and 293TetER/K3R (K3R) stable clones.

2.2.5. BKRF3 was required for EBV genome replication

To monitor the BKRF3 knockout effect on viral DNA replication, EBV genome copy numbers were measured in doxycycline treated p2089, BKRF3STOP and K3R

cells at 72 h postinduction, using quantitative real-time PCR. After doxycycline induction, immunoblotting was performed to confirm that BKRF3 was detectable in p2089 and K3R stable cells, but not in BKRF3STOP cells (Fig. 10A). Simultaneously, induction of Rta successfully turned on viral lytic protein expression in p2089, BKRF3STOP and K3R cells, including Zta, BMRF1, BGLF4, BALF5 and gp350/220 (Fig. 10A). The EBV genome copy numbers increased 17- and 28-folds in p2089 PL1 and PL2 cells, respectively, at 72 h postinduction, whereas EBV DNA copy numbers did not significantly increased at 72 h postinduction in all BKRF3STOP pool clones (Fig. 10B, lanes 1 to 10). In the BKRF3 revertant (K3R) clones, EBV genome copy number increased 18-, 44- and 23-folds in K3R PL5, PL6 and PL14 cells, respectively, at 72 h postinduction, indicating that BKRF3 contributes to viral DNA replication and there are no additional mutations in BKRF3STOP (Fig. 10B, lanes 11 to 16). Notably, the expression of cellular UNG2 was significantly reduced at 72 h postinduction in p2089, BKRF3STOP and K3R cells (Fig. 10A), very likely due to virus-mediated host shut off effects. Thus BKRF3 may compensate cellular UNG2 to benefit EBV replication at this time point.

2.2.6. The interaction of BKRF3 with viral proteins in replication complexes promoted EBV DNA replication through enzymatic activity independent pathways

In this study, we found that the aa 81 to 255 of BKRF3 which contains catalytic domain and DNA binding domain was crucial for association with other viral replicating components. To determine whether BKRF3 plays other roles, in addition to providing the UDG activity in EBV DNA replication, *trans*-complementation assays

were performed in BKRF3STOP cells with wild-type or mutant BKRF3 expression plasmids. To this end, two Flag-BKRF3 mutants were generated, one mutated at the catalytic site (Q90L,D91N) and the other at the leucine loop (H213L) (Fig. 11A and Geoui et al. 2007). As indicated by the immunoblot, viral lytic protein expression was comparable in cells complemented with plasmids expressing wild-type or mutant BKRF3 (Fig. 11B). In the immunoprecipitation-UDG assay, the UDG activity of BKRF3 was normalized with the relative fold of immunoprecipitated HA-tagged BKRF3. The UDG activity of wild-type BKRF3 increased after lytic induction (Fig. 11C, lanes 5 and 6); whereas, the enzymatically dead BKRF3(Q90L,D91N) failed to remove uracils (Fig. 11C, lanes 7 and 8). Interestingly, the leucine loop mutant BKRF3(H213L) which contains wild-type catalytic domain also defected in UDG activity (Fig. 11C, lanes 9 and 10). According to the 3D structural analyses of BKRF3 and human UNG (Geoui et al. 2007, Roberts et al. 2012), the leucine loop of UDG is within its DNA-contacting region (resides 213 to 229 of BKRF3). Therefore, single-stranded DNA cellulose chromatography was performed to determine whether the substitution of histidine 213 with leucine affects the general DNA binding ability of BKRF3. After binding of *in vitro*-transcribed/translated wild-type or mutant HA-BKRF3 to the DNA cellulose, proteins were eluted with step gradients of NaCl to indicate their relative DNA binding abilities (Fig. 11D). The peaks of both wild-type BKRF3 and BKRF3(H213L) were mostly detected in the 0.2 M NaCl fractions, indicating the general DNA binding affinity of BKRF3(H213L) is very close to that of the wild-type and another mechanism is involved in the inability of BKRF3(H213L) to remove uracil base. On the other hand, BKRF3(Q90L,D91N) was majorly eluted in the

0.3 M NaCl fraction (Fig. 11D). According to PyMOL 3D structure prediction, mutation sites of BKRF3(Q90L,D91N) are close to β1 sheet of BKRF3 and may create a conformational change of BKRF3 (Fig. 12), leading to a slight increase of DNA binding affinity.

Consequently, the EBV genome copy number was monitored to verify the biological function of BKRF3 in BKRF3STOP cells after complementation. Compared to that of vector control transfected cells, EBV genome copy numbers increased 29-, 34- and 33-folds at 60 h postinduction in BKRF3STOP PL1, PL5 and PL11 cells, respectively, with the complementation of wild-type BKRF3, indicating that BKRF3 is crucial for EBV replication (Fig. 11E, lanes 3, 8 and 13). Notably, complementation of enzymatically dead BKRF3(Q90L,D91N) also increased viral DNA copies 22-, 31- and 18- folds in doxycycline-treated BKRF3STOP PL1, PL5 and PL11 cells, respectively, (Fig. 11E, lanes 4, 9 and 14), suggesting that BKRF3 plays an essential role in the DNA replication process, in addition to its UDG activity. In cells complemented with the leucine loop mutant BKRF3(H213L), viral DNA increased 5-, 10- and 6-folds in doxycycline treated BKRF3STOP PL1, PL5 and PL11 cells, respectively (Fig. 11E, lanes 5, 10 and 15). Leucine loops of gammaherpesviral UDGs are seven-amino acid longer than that of alpha- and betaherpesviruses (Fig. 11A). We found the mutation of histidine 213 in BKRF3 attenuated its ability to rescue viral DNA replication in BKRF3STOP cells, indicating the leucine loop of BKRF3 is important for viral DNA replication. Thus data here suggest proper overall structure, but not the enzyme activity, of BKRF3 contributes more to EBV genome replication in our current system.

To determine whether the recruitment of BKRF3 to the viral replication

compartment was affected in BKRF3 mutants, immunofluorescence staining was performed to visualize the co-localization of BKRF3 with DNA polymerase BALF5 in NA cells. With the transfection of Rta expression plasmid, the colocalization intensity of BKRF3(H213L) with BALF5 was weaker than that of WT BKRF3 in reactivated NA cells in confocal analysis (Fig. 13). Consistently, the amounts of HA-BKRF3(H213L) associated Rta and BALF5 were reduced (Fig. 14A, lanes 7 and 8). BKRF3(H213L) still interacted with BMRF1 for its nuclear localization (Fig. 14B), however its targeting to DNA replication complexes was attenuated. Interestingly, the amount of BALF5 DNA polymerase being coimmunoprecipitated with HA-BKRF3(Q90L,D91N) was about 2-fold higher than that of WT BKRF3 (Fig. 14A, lanes 4 and 6). In the middle panels of Fig. 13, more distinct colocalization signals of HA-BKRF3(Q90L,D91N) and BALF5 were also observed. Because both Rta and BALF5 were mapped to interact with the C terminus of BKRF3 (Fig. 7), it is possible that mutation of H213L affects the ability of BKRF3 to interact with these proteins. Overall, data here suggest that the interaction of BKRF3 with viral proteins in replication compartments correlated with its ability to support viral DNA replication.

2.3. Conclusion

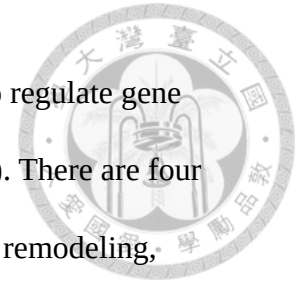
Herpesviral DNA replication-associated enzymes have been considered good antiviral targets. The intranuclear viral DNA replication compartments contain multiple viral and cellular DNA replication and repair enzymes and chromatin modifiers that can coordinately function together for efficient virus replication. In this study, we revealed how BKRF3 is recruited into the nucleus by viral DNA polymerase processivity factor BMRF1 (Fig. 5 and 6). We also showed the regulation of the enzyme activity of BKRF3 and its interaction with viral DNA replication protein complexes and viral DNA replication (Fig. 4, 7 and 8). The inability of the BKRF3STOP bacmid to replicate following lytic induction indicates that BKRF3 is crucial for viral DNA replication (Fig. 9 and 10). The distinct outcomes of complementation with BKRF3(Q90L,D91N) and BKRF3(H213L) suggest the recruitment function of BKRF3 via its C-terminal leucine loop is important for EBV replication (Fig. 11 to 14).

Chapter 3: Transcriptional regulation activities of EBV DNA polymerase processivity factor BMRF1 on viral gene expression

3.1. Introduction

In lytic phase of EBV replication, BMRF1 has dual functions in viral DNA replication and viral gene transcription (Tsurumi 1993, Zhang et al. 1996, Zhang et al. 1997, Neuhierl and Delecluse 2006, Nakayama et al. 2009). BMRF1 associates with viral DNA polymerase BALF5 to stabilize the DNA binding ability of BALF5 (Tsurumi et al. 1993, Tsurumi et al. 1993). By electron microscopy analysis, BMRF1 forms a ring-shaped hexamer structure as well as the clamping protein (Makhov et al. 2004). In addition to accessory activity, BMRF1 serves as a transcription activator to transactivate viral oriLyt BHLF1 promoter through GC-rich SP1/ZBP89 binding element (Zhang et al. 1996, Zhang et al. 1997). Additionally, BMRF1 activates transcription of cellular gastrin gene which was mediated by two SP1/ZBP-89 binding sites in the gastrin promoter (Holley-Guthrie et al. 2005). The amino acid 379 to 388 of BMRF1 has been characterized as a transactivation domain (Zhang et al. 1999). However, the BMRF1-regulated viral/cellular genes are unclear until now. Besides the transactivator activity, BMRF1 also interacts with Zta to bind to the promoter of ssDNA binding protein BALF2 and to enhance the transactivation activity of Zta on BALF2 promoter (Nakayama et al. 2009). Therefore, BMRF1 may regulate viral or host gene expression through interacting with transcriptional regulators.

In mammalian cells, transcription factors work in concert with



chromatin-regulatory factors which alter local chromatin structure to regulate gene expression (Levine and Tjian 2003, Bird 2007, Goldberg et al. 2007). There are four types of epigenetic regulations, including ATP-dependent chromatin remodeling, histone modification, DNA methylation and non-coding RNAs (Dawson and Kouzarides 2012). Alteration of chromatin structure by ATP-dependent chromatin remodeling complexes is pondered a critical step in transcriptional regulation of eukaryotic genes. These chromatin-modifiers use the energy from ATP hydrolysis to actively change the chromatin structure (Johnson et al. 2005). Based on the defining ATPase, chromatin remodeling complexes are divided into four groups as the SWI/SNF (SWItch/Sucrose Non-Fermentable), ISWI (Imitation SWI), CHD (Chromodomain Helicase DNA binding protein 4), and INO80 families of remodelers (Morrison and Shen 2009, Hargreaves and Crabtree 2011).

Human SWI/SNF complex comprises 11 to 15 protein subunits using either BRG1 (SWI/SNF-Related Matrix-Associated Actin-Dependent Regulator Of Chromatin Subfamily A Member 4, SMARCA4) or hBRM (SMARCA2) as the ATPase subunit (Reisman et al. 2009, Wilson and Roberts 2011). The subunit proteins (BRG1-associated factors) contain BAF170, BAF155, BAF47, BAF60, BAF57, BAF53 and actin. BRG1 and hBRM share a high degree of sequence identity (74%) and display similar biochemical activities in vitro (Khavari et al. 1993, Phelan et al. 1999). The SWI/SNF complex facilitates both of gene activation and repression and contributes to the regulation of cell lineage development (Narlikar et al. 2002, Martens and Winston 2003). BRG1 can also assemble with transcription factors and histone-modifying enzyme complexes to activate or repress nuclear processes including transcription

initiation and elongation and DNA replication (Kitagawa et al. 2003, Xu et al. 2004, Trotter and Archer 2008). BRG1 interacts with p53 and is required for the activation of p53-mediated transcription (Lee et al. 2002). In addition, BRG1 regulates gene transcription of matrix metalloproteinase-2 (MMP-2) by modifying the DNA-binding capacities of SP1 and SP3 (Ma et al. 2004).

During herpesvirus infection, viruses manipulate viral chromatin structure to ensure viral genome propagation. Several different histone deacetylases, including HDAC-1, -5, and -7, are recruited to the ORF50 promoter in KSHV latently infected cells. The sodium butyrate (SB) treatment led to the rapid association of chromatin remodeler INI1/SNF5 (BAF47/SMARCB1) with the ORF50 promoter (Lu et al. 2003). In addition, CBP, BRG1, and thyroid hormone receptor-associated protein (TRAP)/Mediator complexes associate with ORF50 to facilitate KSHV lytic reactivation (Gwack et al. 2003). The interaction of EBV latent protein EBNA-2 with INI1/SNF5 may manipulate chromatin structure of target genes (Wu et al. 1996). Therefore, the SWI/SNF complex serves as a chromatin regulator or a transcriptional mediator in herpesvirus infection. However, the roles of SWI/SNF complexes in EBV lytic reactivation need to be elucidated.

In this study, we generated BMRF1 knockout EBV bacmid to investigate the transcription regulatory function of BMRF1 in a whole virus setting. Using the EBV DNA microarray assay to identify the BMRF-regulated viral genes, we found that EBV early lytic genes BaRF1 and BORF2 and late lytic genes BLLF1 (gp350/220), BLLF2, BcLF1 (major capsid protein), BDLF3 (gp150) and BILF2 (gp78/55) were down-regulated in BMRF1 knockout cells. In luciferase reporter assays, BMRF1

expression alone turned on the promoter activity on BcLF1, BDLF3, BLLF1 and BLLF2, but not on BILF2 promoters. In addition, shRNA targeting to SP1 was used to verify whether SP1 is involved in BMRF1-mediated activation on viral late promoters. Depletion of SP1 reduced the BMRF1-mediated transactivation on BDLF3 promoter. On the other hand, BMRF1-associated proteins were analyzed by mass spectrometry-based immunoprecipitation proteomics analysis. Here we demonstrated that BMRF1 associated with BRG1 in the cells and *in vitro*. In reporter assays, the transactivation activity of BMRF1 was reduced on a subset of viral promoters in BRG1 knockdown cells. It is proposed that BMRF1 may cooperate with chromatin modifier BRG1 to regulate EBV gene expression. Interestingly, EBV lytic proteins were up-regulated in BRG1 knockdown 293TetEZ/p2089 cells without lytic induction, suggesting that BRG1 may play an important role in EBV gene expression.

3.2. Results

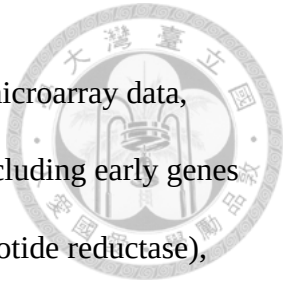
3.2.1. Construction of p2089△BMRF1 and selection of inducible cells

To investigate BMRF1 regulated viral gene expression in the context whole virus system, we constructed p2089△BMRF1 by PCR targeting (Fig. 15A) (Delecluse et al. 1998, Datsenko and Wanner 2000). The BamHI-M fragment size of p2089△BMRF1 was increased from 7980 bp to 8471 bp (Fig. 15B). EBV p2089 or p2089△BMRF1 bacmid was transfected into 293TetEZ or 293TetER cells, the doxycycline inducible cell lines for Zta or Rta expression (Chen et al. 2009), and cells were selected by hygromycin (100 μg/ml) for one month. Four to six GFP-positive cell colonies were

pooled together to establish the 293TetEZ(R)/p2089 and 293TetEZ(R)/p2089△BMRF1 stable cells. The lytic cycle of Maxi-EBV was induced by doxycycline (Dox) treatment (100 ng/ml) for 48 h, and subsequently the lytic proteins, including Zta, Rta, BMRF1, BALF2 and BGLF4, were detected by western blotting. The results showed that doxycycline treatment triggered EBV immediate early protein Zta or Rta expression, and induced EBV lytic cycle. The expression of BMRF1 was not detected in 293TetEZ(R)/p2089△BMRF1 stable cells (Fig. 15C and D).

3.2.2. Knockout of BMRF1 reduced a subset of viral lytic gene expression

To explore possible BMRF1-mediated regulation of viral gene expression, the BMRF1 knockout bacmid was induced for lytic cycle gene expression and analyzed with our homemade EBV array (Lu et al. 2006). The EBV wild-type or BMRF1 knockout bacmid stably transfected 293TetEZ or 293TetER cell lines were treated with doxycycline for 48 h. After lytic induction, total RNA from control or doxycycline treated cells were harvested and reverse-transcripted and labeled with biotin. Subsequently, the biotin-labeled cDNA were used to probe EBV DNA microarray. The lytic activation folds of viral genes were normalized with non-induced control and shown as a heat map representing log2-fold changes of a knockout virus containing cell line versus paired wild-type controls (Fig. 16A and B). The induction fold of BMRF1-regulated EBV genes in Rta inducible cells (293TetER/△BMRF1) were shown from down-regulation to up-regulation, and the gene regulation fold in Zta inducible cells (293TetEZ/△BMRF1) was shown side by side with that in Rta inducible cells. We found that knockout of BMRF1 down-regulated some of Rta-regulated lytic



genes in both Rta and Zta-inducible cells (Table2). Consistent with microarray data, some of EBV lytic genes decreased in 293TetER/△BMRF1 cells, including early genes BORF2 (RR1, ribonucleotide reductase) and BaRF1 (RR2, ribonucleotide reductase), BLLF1 (gp350/220), BLLF2 (uncharacterized protein), BcLF1 (major capsid protein), BDLF3 (gp150) and BILF2 (gp78/55) detected in RT-PCR analysis (Fig. 16C). Simultaneously, expression of late genes BSRF1 (uncharacterized protein) and BDRF1 (packaging protein) which were not affected in the microarray assay were confirmed by RT-PCR (Fig. 16D). Because BMRF1 is required for DNA replication, therefore we confirmed the EBV DNA copy number of BMRF1 knockout cells after lytic induction. Comparison with wild-type cells, viral DNA replication was defective in BMRF1 knockout cells (Fig. 17A and B, lane 2). Furthermore, complementation of BMRF1 rescued viral DNA replication in BMRF1 knockout cells (Fig. 17A and B, lane 3) that is similar to previous studies (Tsurumi et al. 1993, Kiehl and Dorsky 1995, Neuhierl and Delecluse 2006). Previous studies reported that most of EBV late gene expression was repressed when viral DNA replication was inhibited (Summers and Klein 1976, Rickinson and Epstein 1978). Indeed, viral late gene transcripts BcLF1, BLLF1 and BLLF2 were not obviously increased in BMRF1 knockout cells (Fig. 17C, lane 2) and were significantly increased more than 40-folds in BMRF1 complemented cells (Fig. 17C, lane3). However, we found a subset of late gene expression was not affected in BMRF1 knockout cells (Fig. 16D), indicating the down regulation of late genes in BMRF1 knockout cell was not caused completely by the defective of viral DNA replication.

3.2.3. BMRF1 functions as a transactivator or a coactivator to turn on certain Rta responsive late gene promoters

To determine whether BMRF1 can turn on promoter activities of those late genes which were down-regulated in BMRF1 knockout cells, the transactivation activity of BMRF1 on BcLF1, BDLF3, BLLF1, BLLF2 or BILF2 promoter was detected by luciferase assay. As shown in Fig. 18, BMRF1 expression alone directly turned on the promoter activities of EBV late genes BcLF1, BDLF3, BLLF1 and BLLF2, but not that of BILF2, in a dose dependent manner. Previously, it was reported that Rta but not Zta turns on the promoter activities on late gene promoters, including BLLF1, BcLF1, BDLF3 and BILF2 etc.(Chua et al. 2007). Therefore, we want to dissect whether BMRF1 contributes to Rta-mediated transactivation activity on those late gene promoters. Indeed, the expression of Zta was unable to turn on the reporter activity of BLLF1 promoter in 293T cells (Fig. 19A). The expression of BMRF1 enhanced Rta-mediated luciferase activities in a dose dependent manner on BcLF1, BDLF3 and BLLF2 promoters, and the enhancement was disappeared when the transactivation domain of BMRF1 was deleted (Fig. 19B to C). However, BMRF1 did not significantly enhance the promoter activity of BILF2 in reporter assays (Fig. 19E). It is possible that the BMRF1 mediated regulatory region was not included in current reporter plasmid construct or the activation of BILF2 promoter by BMRF1 is through an indirect pathway.

3.2.4. BMRF1 turned on the promoter of BDLF3 through an SP1-dependent pathway

Previous studies showed that BMRF1 regulates viral BHLF1 and host gastrin gene promoters through an SP1/ZBP89 binding site (Zhang et al. 1997, Holley-Guthrie et al. 2005). The promoter regions of BLLF1, BLLF2, BDLF3 and BcLF1 contain many putative SP1 binding sites, suggesting BMRF1 may regulate these promoters in an SP1-dependent manner. To verify this possibility, the reporter assay was performed in SP1-knockdown HEK293 cells. Interestingly, knockdown of SP1 dramatically repressed the activation fold of BHLF1 and BDLF3 promoters by BMRF1, but not that of BLLF1 and BcLF1 promoters (Fig. 20). According to above results, BMRF1 regulated promoter activation of BDLF3 through an SP1 dependent pathway. Besides SP1, it is possible that BMRF1 may coordinate with other cellular factor(s) to regulate the expression of BcLF1 and BLLF1.

3.2.5. BMRF1 associates with cellular factors involved in DNA replication, chromatin remodeling and RNA splicing

Because the promoter specificity of BMRF1-mediated gene activation remains unclear, therefore we want to know whether BMRF1 cooperates with other factor(s) to regulate gene expression. The HA-tagged BMRF1 expressed 293TetEZ/△BMRF1 cells was treated with doxycycline to induce lytic reactivation. Subsequently, HA-tagged BMRF1 associated complexes were immunoprecipitated and separated by SDS-PAGE. As reported previously (Zeng et al. 1997, Nakayama et al. 2009), BMRF1 associated with Zta and BALF2 after lytic induction (Fig. 21A, lane 3). As shown in Fig. 21B,

BMRF1 associated with a number of proteins in BMRF1 knockout cells with or without lytic induction. We found the intensity of indicated band-1 with BMRF1 interacting proteins was decreased, whereas the intensity of indicated band-2, -3 and -4 with BMRF1 interacting proteins were increased after induction, implying the proteins associated with BMRF1 or disassociated from BMRF1 may affect lytic cycle progression. Therefore, the indicated bands with increased or decreased intensities after lytic induction were sliced and analyzed by mass spectrometry (Fig. 21B). BMRF1 interacting proteins sorted from indicated bands-1 and -2 were summarized in Table 3. In addition, the major BMRF1-interacting proteins indicated as band-3 and -4 are hyper- and hypo-phosphorylated BMRF1, respectively (Fig. 21B). The BMRF1 interacting protein candidates which may increase the interaction affinity with BMRF1 indicated as band-2, including DNA replication/repair associated factors, chromatin remodeling factors and RNA binding proteins. In contrast, the BMRF1 interacting protein candidates which may decrease the interaction affinity with BMRF1 indicated as band-1, including histone deacetylase 2, RNA binding proteins and protein folding related protein CCT8 (Table.3). Previous studies demonstrated chromatin remodelers participate in regulating herpesvirus infection (Wu et al. 1996, Gwack et al. 2003, Lu et al. 2003). BRG1, one of BMRF1 associated chromatin remodelers, is important for KSHV reactivation (Lu et al. 2003). Because BRG1 regulates chromatin structure to activate or repress gene transcription (Wilson and Roberts 2011), we examined whether BRG1 is involved in BMRF1-regulated gene expression.

3.2.6. BMRF1 interacts with chromatin modifier BRG1 in a DNA independent manner.

To confirm the association of BMRF1 with BRG1 identified by IP-spectrometry, the co-localization of BMRF1 and BRG1 was observed by immunofluorescence staining. HeLa cells were transfected with Flag-BRG1 and HA-BMRF1 for 24 h. The nuclear pattern of Flag-BRG1 was partially colocalized with that of HA-BMRF1 (Fig. 22A). Indeed, endogenous BRG1 colocalized with BMRF1 during EBV reactivation in NA cells was observed by confocal microscopy (Fig. 22B). After lytic induction, BMRF1 was detected in BRG1-associated immunocomplexes in Rta-transfected NA cells (Fig. 23A). Furthermore, cell lysates harvested from 293T cells were used for GST pull down assay to determine the interaction of BRG1 and GST-BMRF1. We found that BRG1 associated with GST-BMRF1 in vitro (Fig. 23B). To determine whether the interaction of BMRF1 and BRG1 is DNA-dependent, the cell lysates harvested from Rta-reactivated NA cells were pretreated with ethidium bromide (EtBr) to disrupt DNA-dependent protein associations. The result showed that BMRF1 still associated with BRG1 after EtBr pretreatment, suggesting the interaction between BMRF1 and BRG1 is independent of DNA binding (Fig. 23C). Moreover, the deletion of transactivation domain of BMRF1 abolished the interaction ability with BRG1, indicating the transactivation domain of BMRF1 is required for the interaction to BRG1 (Fig. 23D).

3.2.7. BRG1 contributes to BMRF1 transactivation of BHLF1, BLLF1 and BcLF1 promoters.

As the result showed that BMRF1 interacts with BRG1 during lytic reactivation, we examined whether BRG1 is involved in the BMRF1-mediated activation of BLLF1 and BcLF1 promoters. In reporter assays, knockdown of BRG1 down regulated the transactivation activity of BMRF1 on the BHLF1, BLLF1 and BcLF1, but not that on BDLF3 promoter (Fig. 24). Therefore, BRG1 not only regulates BLLF1 and BcLF1 late gene promoters but also regulates the oriLyt BHLF1 promoter, indicating the BRG1 may regulate multiple promoter regions in EBV.

3.2.8. Chromatin modifier BRG1 probably sustained the latency of EBV.

It has been reported that BRG1 associates with viral proteins of KSHV to manipulate chromosome structure for virus propagation in host cells (Gwack et al. 2003, Lu et al. 2003). Therefore, it is possible that BRG1 may contribute to the reactivation of EBV. To verify this possibility, 293TetZE/p2089 wild-type cells were transduced with shBRG1 and induced EBV lytic reactivation with or without doxycycline. At 60 h postinduction, viral proteins were detected by western blotting analysis. We found that knockdown of BRG1 induced the expression of viral lytic proteins, including immediate early, early and late proteins, suggesting BRG1 may be involved in the latent-lytic switch of EBV (Fig. 25).

3.3. Conclusion

EBV BMRF1 has multiple functions to regulate virus replication in lytic cycle. The first function is to act as a DNA polymerase accessory factor in viral DNA replication; the second function is to participate in nuclear targeting of BKRF3; the third function of BMRF1 acts as a transcriptional activator to activate viral gene promoters BHLF1 and BALF2. Here we demonstrated that BMRF1 also enhances a subset of EBV gene expression. In EBV DNA microarray data, the BMRF1-regulated gene candidates including BaRF1, BORF2, BLLF1, BLLF2, BILF2, BDLF3 and BcLF1, are defined as Rta-mediated genes which contain a number of Rta responsive elements (RRE, 5'-GNGGN_9CCNG-3') on the promoter regions (Fig. 16 and 17). The reporter assay showed that BMRF1 alone activated Rta responsive genes BLLF2, BDLF3, BcLF1 and BLLF1, but not BILF2 (Fig. 18). In the presence of Rta, BMRF1 enhanced the Rta-mediated transactivation with an additive effect on BcLF1, BLLF2 and BDLF3 promoters (Fig. 19). Using TFSEARCH program to search transcription factor binding sites, we found there are a number of SP1/ZBP89 binding sites on those promoters (Fig. 20A) Previous studies have shown that BMRF1 activates BHLF1 and gastrin promoters through SP1/ZBP binding sites (Zhang et al. 1996, Holley-Guthrie et al. 2005). Knockdown of SP1 blocked BMRF1-mediated gene activation of BDLF3 as well as that of BHLF1, suggesting that BMRF1 activates BDLF3 gene expression through an SP1 dependent pathway (Fig. 20E). In addition, BMRF1 also interacted with cellular chromatin modifier BRG1 in the cells and *in vitro* (Fig. 22 and 23). Depletion of BRG1 attenuated the BMRF1-mediated activation fold of late genes BcLF1 and BLLF1, suggesting BRG1 is involved in the transcriptional regulation of BMRF1 (Fig. 24).

Based on immunoprecipitation-mass spectrometry analysis data (Table 3), BMRF1 also interacts with a number of cellular factors which regulate gene expression such as chromatin remodelers, DNA replication/repair proteins, and RNA binding/slicing proteins (Fig. 21 and Table 3). First are chromatin remodeling factors, including BRG1, CHD4 (Chromodomain-helicase-DNA-binding protein 4), HDAC2, etc., modulate chromatin architecture to control gene expression (de la Serna et al. 2006, Denslow and Wade 2007, Trotter and Archer 2008). Secondly, DNA replication/repair/recombination proteins such as CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase) and topoisomerase IIα are required for pyrimidine biosynthesis and productive RNA synthesis on chromatin templates, respectively (Mondal and Parvin 2001, Grande-Garcia et al. 2014). Finally, the RNA binding protein (polypyrimidine tract-binding protein 1, PTBP1) is involved in regulating the stability of mRNA (Kosinski et al. 2003). Therefore, we propose that BMRF1 may regulate gene activation through interacting with cellular transcription regulatory proteins. Taken together, BMRF1 can utilize distinct host transcriptional regulator to control the expression of different EBV genes during lytic cycle.

Chapter 4: Discussion

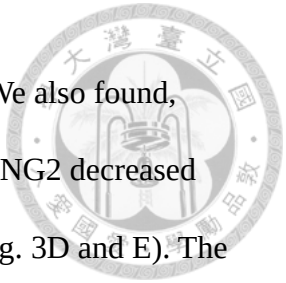
4.1. BKRF3 participates in viral DNA replication compartments during EBV reactivation

Catalytic activities of both cellular UDG UNG2 and viral UDGs contribute to herpesviral DNA replication. To ensure that the enzyme activity executes at the right time and the right place in DNA replication forks, complex formation with other components in the DNA replication machinery provides an important regulation for UDG function. In this study, we provide the mechanism for EBV UDG BKRF3 nuclear targeting and the interacting domains of BKRF3 with viral DNA replication proteins. Through knockout and complementation approaches, we further demonstrate that in addition to UDG activity, the interaction of BKRF3 with viral proteins in the replication compartment is crucial for efficient viral DNA replication.

4.1.1. EBV uracil-DNA glycosylase BKRF3 expresses and associates with BMRF1 and other replication proteins in the nucleus during lytic cycle

4.1.1.1. The up-regulated expression of BKRF3 accompanies with down-regulation of cellular UNG2.

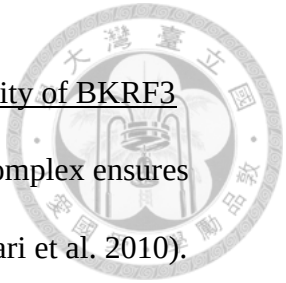
The biological function of uracil-DNA glycosylase (UDG) is to prevent mutagenesis by eliminating uracil from DNA molecules and initiating the BER pathway. In chapter 2, we observed that viral UDG BKRF3 was translocated from the cytoplasm into the nucleus upon lytic induction and colocalized with the DNA polymerase BALF5



in both NPC NA cells and 293-derived EREV8 cells (Fig. 3A to C). We also found, upon the induction of lytic replication, that the expression levels of UNG2 decreased along with increasing expression of viral lytic replication proteins (Fig. 3D and E). The exact mechanism leading to UNG decrease during virus replication is not clear. However, the expression of UNG2 was reported to peak in and throughout S phase and then decline to undetectable levels until the next S phase through the ubiquitin-proteasome pathway (Hardeland et al. 2007). Taking into account that EBV replication causes cell cycle arrest at the G1/S transition without cellular DNA replication (Guo et al. 2010, Chen et al. 2011), we suspect the decrease of UNG2 is caused by downregulated transcription or increased protein degradation.

4.1.1.2. The mechanism of BKRF3 nuclear targeting during EBV reactivation

Unlike cellular UNG2, which contains a unique NLS as well as subnuclear targeting signals (Otterlei et al. 1998), we found that the interaction of BKRF3 with viral DNA polymerase processivity factor BMRF1 promoted its nuclear transport (Fig. 5). The nuclear targeting of BKRF3 is similar to that of HCMV UL114, which associates with UL44, the homolog of BMRF1 (Prichard et al. 2005), suggesting interactions with nuclear replicating proteins are required for herpesviral UDGs to participate in the viral replication compartment. The nuclear targeting of BALF5 was recently reported to be dependent on BMRF1 and cellular chaperone protein Hsp90 (Kawashima et al. 2013). It is not clear whether other cellular proteins are required for BMRF1-dependent nuclear targeting of BKRF3.



4.1.1.3. Interaction with replication proteins augments the UDG activity of BKRF3

Interaction of cellular UNG2 with PCNA and RPA replicating complex ensures post-replicative repair of misincorporated uracil (U·A) in DNA (Akbari et al. 2010). Similarly, the association between UDGs and viral DNA polymerases to function in replication-coupled base excision repair has been described for HSV-1 and HCMV (Bogani et al. 2010, Strang and Coen 2010). HCMV UL44 facilitates loading of UL114 onto DNA and promotes UL114 to remove uracil from DNA (Ranneberg-Nilsen et al. 2008). In our study, BKRF3 interacted with the immediate-early transactivator Rta, DNA polymerase BALF5, and DNA polymerase processivity factor BMRF1 in coimmunoprecipitation and GST pulldown assays (Fig. 4, 6 and 7). We suggest the interaction with BALF5 and BMRF1 helps BKRF3 to form complexes with other replication proteins to enhance UDG activity of BKRF3 and carry out repairs at DNA replication loci. Because Rta was found to promote the nuclear targeting of the DNA primase-associated factor BBLF2/3, which is the linker for proteins targeting viral DNA replication origin (Liao et al. 2005), it is possible that Rta also helps the stabilization of BKRF3 to the DNA replication complex through network interactions with other viral replication proteins. Furthermore, the UDG activity of HA-BKRF3 was stimulated when it formed complexes with other viral DNA replication proteins (Fig. 8). In this way, viral UDG activity may be regulated to function along with viral DNA replication, avoiding nonspecific DNA damages.

4.1.2. The presence of BKRF3 is essential for EBV lytic DNA replication.

A search through the literature found that the UDG homologs are dispensable in

alphaherpesviruses for infection of actively growing fibroblasts but are essential for infection and reactivation in the murine nervous system (Pyles and Thompson 1994, Bogani et al. 2010). The UDG of HCMV, UL114, is required for full virus replication in a culture system. Viral replication of the HCMV UL114 mutant is retarded in quiescent fibroblasts but proceeded smoothly in actively growing fibroblasts (Courcelle et al. 2001). Additionally, using a random signature tagged mutagenesis approach to select the viral genes essential for the murine gammaherpesvirus 68 life cycle, Song et al. found viral UDG ORF46 is not essential for viral replication in a mouse fibroblast cell line (Song et al. 2005). These observations suggest that cellular UDG activity is able to compensate for viral UDG deficiency to a certain extent. Previously, we found viral DNA replication was decreased about 10 to 20% at 48 h postinduction when BKRF3 was knocked down by siRNA (Lu et al. 2007). It is possible that residual BKRF3 was sufficient to support EBV DNA replication complex formation in that setting. In this study, we demonstrated cellular UNG2 was undetectable at 60 h post-EBV reactivation, while expression of BKRF3 gradually increased and the total UDG activity was sustained in NA cells (Fig. 3E). Thus, the BKRF3 UDG activity may function in cells with low levels of UNG2 expression.

Notably, the presence of BKRF3 in the viral DNA replication compartment is correlated with its function in DNA replication. In bacmid experiments, we found the EBV DNA copy number significantly increased about 20- to 40-fold following the induction of Rta by doxycycline in p2089 and in K3R revertant cells, but not in BKRF3STOP cells, at 72 h postinduction (Fig. 10). In the sequence alignment, the extended leucine loop at the C-terminal region is conserved among gammaherpesviruses

(Fig. 11A), suggesting functions in facilitating viral DNA replication. BKRF3STOP virus, which was inserted with a stop codon cassette at aa 73 of BKRF3, did not replicate after lytic induction. However, MHV-68 ORF46 mutant virus, which was inserted with a transposon at aa 103 of the viral UDG, still produced infectious viruses (Song et al. 2005). We suspect that BKRF3 plays a unique role in EBV DNA replication or the residual fragment of truncated UDG in MHV-68 still facilitates viral DNA replication. Nevertheless, expression levels of UNG2 in virus-replicating cells may affect the outcome of viral UDG defective viruses. Thus, it would be interesting to address whether UNG2 also participates in the viral DNA replication compartment and tethers other cellular DNA replication factors to enhance viral DNA replication.

4.1.3. The protein interaction ability of BKRF3, but not the enzymatic activity of that, is critical for lytic reactivation

Complementary expression of both the wild-type and two BKRF3 mutants in BKRF3STOP cells further demonstrated the contribution of different functional domains of BKRF3 in viral DNA replication. Previously, we showed that blocking cellular and viral UDG activity by the inhibitor Ugi decreases 50% of EBV DNA replication. Here, the fact that enzymatically dead mutant BKRF3(Q90L,D91N) restored EBV genome replication in BKRF3STOP cells at a slightly lower level than WT BKRF3 strongly suggests that BKRF3 has a function other than its enzymatic activity for EBV genome replication (Fig. 11E). With nuclear distribution upon lytic induction, BKRF3(H213L) interacts with proteins in the viral DNA replication complex less efficiently, as revealed by immunofluorescence and coimmunoprecipitation

experiments (Fig. 13 and 14A). Data here hint that precise associations with other components in the complex affect the enzymatic activities and function(s) of BKRF3. It is possible that BKRF3 promotes a conformational change of BALF5/BMRF1 complexes to enhance DNA synthesis. Such interactions also ensure viral UDG is recruited to viral DNA replication forks before DNA synthesis. Regulation through specific interaction with DNA binding proteins were founded in cellular and viral UDGs. For example, UNG2 directly interacts with XRCC1 to enhance the repair efficiency of the XRCC1-mediated repair pathway (Chen et al. 2005, Akbari et al. 2010). Likewise, the HCMV UDG, UL114, was found to interact with the chromatin remodeling factor SMARCB1 and to participate in the recruitment of the chromatin remodeling complex onto replication foci (Ranneberg-Nilsen et al. 2008). Although no BKRF3-interacting cellular proteins were identified in two previous yeast-two hybrid screening studies (Calderwood et al. 2007, Gulbahce et al. 2012), it is still possible that BKRF3 also recruits the cellular machinery to support viral DNA replication.

4.2. Transcriptional regulation of BMRF1 on EBV gene expression

BMRF1 is capable of facilitating both DNA replication and gene transcription in EBV lytic cycle. The DNA polymerase processivity ability of BMRF1 has been well characterized previously (Tsurumi 1993, Tsurumi et al. 1993, Kiehl and Dorsky 1995). Besides associating with DNA polymerase BALF5 for efficient viral DNA replication, BMRF1 also promotes nuclear targeting of BKRF3 to allow BKRF3 to execute its role in viral DNA replication (chapter 2). In regulating viral gene expression, BMRF1 acts as an activator or a coactivator to regulate early gene promoter of oriLyt BHLF1 and BALF2, respectively (Zhang et al. 1997, Nakayama et al. 2009). Here, we demonstrate that BMRF1 activates EBV late promoters, in addition to regulating early viral promoters. We also found BMRF1 interacts with chromatin remodeling factors during lytic replication. Moreover, the chromatin remodeling factor BRG1 contributes to BMRF1-mediated transcriptional regulation. Therefore, protein-protein interactions may be important for the regulatory functions of BMRF1 in DNA replication and gene expression.

4.2.1 BMRF1 contributes to regulation of late gene transcription in a DNA replication independent manner

In chapter 3, we found that EBV late genes including BcLF1, BLLF1 and BFRF3, were regulated by BMRF1 (Fig. 16 and Table2). Previous studies showed the regulation of late gene expression through a DNA replication dependent manner. During the lytic cycle, EBV DNA production was inhibited by PAA treatment. The PAA treatment of B95.8 showed the total inhibition of viral capsid antigen but not early antigen synthesis,

suggesting that viral DNA replication is a prerequisite for late gene expression (Summers and Klein 1976). However, the replication of the transcriptional template is not essential for expression of late gene BcLF1 and BFRF3 (Serio et al. 1997). In 293 cells, Rta alone still induces late gene BLLF1 expression in Zta-deleted virus without productive EBV DNA replication (Feederle et al. 2000). Interestingly, reduction of BLLF1 transcript was observed in Rta inducible BMRF1 knockout cell with defective EBV DNA replication, implying BMRF1 is important for expression of BLLF1 in Rta-induced lytic reactivation (Fig. 16). In addition, the reporter assay demonstrated that BMRF1 expression alone can transactivate the promoters of BcLF1 and BLLF1 in 293T cells (Fig. 18). Therefore, we suggest that BMRF1 can regulate EBV late gene transcription independent of viral DNA replication.

4.2.2 BMRF1 and Rta act in an additive manner to activate a subset of late promoters

In the presence of Rta, expression of BMRF1 enhances Rta-mediated activation of of BcLF1, BDLF3 and BLLF2 promoters (Fig. 19). Rta was identified as a transactivator to activate some viral late genes, including BcLF1, BDLF3, BILF2, BLLF1, and BLRF2 (Chua et al. 2007). A recent study reported that BMRF1 functions as a coactivator to enhance the Zta-dependent transactivation activity through interacting with Zta (Nakayama et al. 2009). Therefore, it is possible that BMRF1 may serve as a coactivator of Rta-mediated transactivation on certain late promoters. However, here we did not observe BMRF1 interacted with Rta in vitro. In addition, expression of BMRF1 alone sufficiently turned on promoter activities of these

Rta-responsive late genes (Fig. 18). Therefore, we proposed that BMRF1 regulates Rta-responsive EBV late promoters through an Rta-independent pathway.

4.2.3 BMRF1 associates with cellular factors to regulate EBV gene expression

4.2.3.1 BMRF1 transactivates BDLF3 promoter through a SP1 dependent pathway

As shown in Fig. 20E, BMRF1-induced activation of BDLF3 promoter was diminished in SP1 knockdown cell. In the transcriptional regulation of oriLyt BHLF1, BMRF1 induces the promoter activity through interacting with SP1 bound to GC-rich motif within the promoter of BHLF1 (Zhang et al. 1996, Zhang et al. 1997, Chang et al. 2005). Therefore, we propose that BMRF1 regulates BDLF3 promoter through SP1. However, although there are a number of SP1 binding sites within BcLF1 and BLLF1 promoters which mediated by BMRF1 (Fig. 20A), BMRF1 still turned on the promoter activity of BcLF1 and BLLF1 in SP1 knockdown cells in reporter assays. It is possible that BMRF1 may regulate these two promoters through other mechanisms, implying other factors are involved in BMRF1-mediated transactivation.

4.2.3.2 Chromatin regulators may participate in the transcriptional regulation of BMRF1

Chromatin-remodeling enzymes are generally defined into two main classes: those that alter histone-contacts using ATP hydrolysis activity, and those that covalently modify histone proteins (de la Serna et al. 2006). Both classes have important roles in gene regulation. In the IP-mass spectrometry analysis, we found that BMRF1 associated

with two chromatin-remodeling ATPases BRG1 (SMARCA4, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4) and CHD4 (Chromodomain helicase DNA-binding protein 4) (Table 3). In addition, the histone deacetylase HDAC2 was also detected in the BMRF1-associated immunocomplex. It is shown that BMRF1 interacts with BRG1 in vivo and in vitro (Fig. 22 and 23). The interaction between BMRF1 and those chromatin-remodeling factors may provide a mechanism to activate transcription of EBV genes.

4.2.3.2.1 BRG1 is required for BMRF1-mediated activation of BHLF1, BcLF1 and BLLF1 promoters

In EBV-positive NA cells, the interaction between BMRF1 and BRG1 was clarified by confocal and immunoprecipitation assays (Fig. 22B and 23A). In addition, transactivation domain of BMRF1 is crucial for BRG1 interaction (Fig. 23D). The ATPase BRG1 acts as an activator or a repressor in gene regulation through the modulation of chromatin structure (Reisman et al. 2009). In the activation of eukaryotic genes, a nucleosome-depleted region (NDR) located at upstream of transcription start sites (TSS) are exposed for transcription regulators binding. A recent study shows that SWI/SNF functions to establish and to maintain the nucleosome landscape in the regions surrounding TSS (Tolstorukov et al. 2013). Therefore, we considered the transcription regulatory function of BMRF1 may be controlled by BRG1. By luciferase assays using reporter plasmid containing promoter sequence of BcLF1 and BLLF2 and BHLF1, here showed that knockdown of BRG1 repressed the transactivation activity of BMRF1 on these promoter regions (Fig. 24). These results suggest that BRG1 is

involved in the BMRF1-mediated gene regulation to modulate the expression of not only late genes but also early genes. However, the preference for the target promoters of BMRF1-BRG1-regulated genes need to be elucidated.

4.2.3.2.2 Mi-2/NuRD complex, the potential BMRF1-interacting complex, may function as a repressor in EBV gene regulation

In IP-mass spectrometry analysis, chromatin remodelers, including CHD4, RBBP4 (Retinoblastoma binding protein 4) and HDAC2, were detected in BMRF1-associated complexes. CHD4, RBBP4 are components of Mi-2/ nucleosome remodeling and deacetylase (NuRD) complex (Denslow and Wade 2007). CHD4 comprises the catalytic core of NuRD complex and serves as a scaffold for the interaction of other factors such as HDAC1 and HDAC 2, p66α (GATAD2A), p66β (GATAD2B), RBBP4 and RBBP7, MTA1-3 (metastasis- associated gene protein 1 to 3), and MBD2 and MBD3 (methyl-CpG binding domain protein 2 and 3) to form Mi-2/NuRD complex (Tong et al. 1998, Xue et al. 1998, Zhang et al. 1998, Zhang et al. 1999). The Mi-2/NuRD complex is thought to play a role in modifying chromatin structure to repress gene expression (Denslow and Wade 2007). Therefore, we consider that Mi-2/NuRD complex may be required for BMRF1-mediated gene regulation. To clarify the possibility, the interaction between BMRF1 and Mi-2/NuRD complex needs to be confirmed. Furthermore, the transactivation activity of BMRF1 should be determined in Mi-2/NuRD complex knockdown cells to prove our hypothesis.

4.2.3.3 BRG1 mediated regulation of herpesvirus replication

A previous study demonstrated that HSV encoded transcription activator VP16 recruit histone acetyltransferases CBP and p300, chromatin remodeling factor BRG1 and BRM and general transcription factor TFIID and RNA polymerase II to viral promoters of immediate early genes (Herrera and Triezenberg 2004). In addition, KSHV Rta recruits CBP, BRG1 subunit of SWI/SNF and the TRAP/Mediator coactivator to its promoters, and the recruitment of those chromatin modifiers by Rta is crucial for KSHV lytic reactivation (Gwack et al. 2003). Surprisingly, knockdown of BRG1 resulted in EBV spontaneous reactivation in 293TetEZ/p2089 cells (Fig. 25). This result is contrary to the previous studies indicating the BRG1 is essential for viral lytic reactivation in HSV and KSHV infection (Gwack et al. 2003, Herrera and Triezenberg 2004, Wang et al. 2014). It is possible that the SWI/SNF complex may associate with repressors to occupy lytic promoters on the EBV genome to block lytic replication. Another possibility is knockdown of BRG1 may influence host gene expression which alter the microenvironment of host cells, resulting EBV reactivation. Therefore, we proposed that BRG1 is involved in the regulation of EBV reactivation, and the mechanism of BRG1-mediated EBV lytic gene regulation need to be explored.

4.3. The biological functions of BKRF3 and BMRF1 in EBV reactivation

In summary, BKRF3 is required for EBV lytic DNA replication. Besides UDG activity, the interaction of BKRF3 with viral replication-associated proteins is important for lytic DNA replication. In addition to associate with BALF5, here we demonstrated

that BMRF1 interacts with BKRF3 to promote the nuclear targeting of BKRF3 for efficient viral replication. In viral gene regulation, BMRF1 directly activated a subset of viral late promoters in 293T cells, indicating that BMRF1 regulates these late gene promoters through viral DNA replication independent pathway. Chromatin modifier BRG1 which interacts with BMRF1 may be a critical modulator of transcriptional regulation in EBV lytic gene expression.

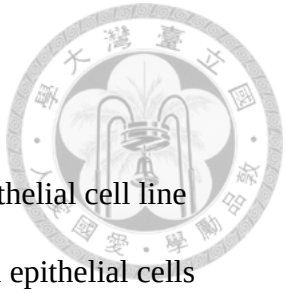
Chapter 5: Materials and Methods

5.1. Plasmids

The full-length BKRF3 PCR products were amplified by primers: 5'-CGGGATCCATGGCA TCGCGGGGGC-3' and 5'- CGGGATCCCTACAGCCTCCAA TCTATC-3' using Akata cell lysates as the template. BKRF3 products were digested with BamHI and were cloned into the BamHI site of pCMV-Tag2B (Stratagene) to generate pFlag-BKRF3 (pNC1). To generate BKRF3 enzymatically dead plasmid pFlag-BKRF3(Q90L,D91N) (pCYC11) or BKRF3 leucine loop mutant plasmid pFlag-BKRF3(H213L) (pCYC12), single primer mutagenesis (Makarova et al. 2000) was performed using the primer 5'- TGGTTATTTTGGGCCTCAACCCCTATCACG GGG-3' or 5'- TTCTGACCTCTCAGCTTCCCTCTCCCCTGGC-3'. In addition, these three BKRF3 constructs were individually sub-cloned into pHY25 plasmid, which is a pSG5 (Stratagene) derivative inserted with the HA sequence and multiple cloning sites, to generate HA-BKRF3 (pIH4), HA-BKRF3(Q90L,D91N) (pIH5) and HA-BKRF3(H213L) (pIH6). For GST pull-down assay, BKRF3 was sub-cloned into EcoRI site of pGEX-4T1 (GE Healthcare) to generate pGEX-4T1-BKRF3 (pSMC8). The pGEX-4T1-BKRF3mts, including GST-d(1-30) (pSMC9), GST-d(28-83) (pSMC10), GST-d(81-166) (pSMC11) and GST-d(164-255) (pSMC12), were constructed by single primer mutagenesis using primers LMRC654 (5'-TCCCCGGAA TTCATGCTCCCCGACTTATGG-3'), LMRC655 (5'-GGTGTGAAAGGAGAAAATG ACCCCTCTGATATTAAG-3'), LMRC656 (5'-CTGGGCCCGCTTTTGCTGGGCGT

GGTTTACTG-3’) and LMRC657 (5’-GCCCGGCTCGCACGCATAGGAATTCCCGG GTC-3’). GST-BMRF1 plasmid (pYPW85) was generated by cloning BamHI- EcoRI BMRF1 fragment into pGEX4T1 vector (Yang et al. 2008). The pCR3.1-BMRF1 expression plasmid (pYPW88) is a subclone of pYPW21 (Yang et al. 2008) using primer LMRC439 (5’-CCGGGATCCATGGAAACCACTCAGACTCT-3’) and LMRC 440 (5’-CGCGAATTCTTAAATGAGGGGGTTAAAGGC-3’). To construct the deletion clone of pCR3.1-BMRF1 (d379-383, △TA), single primer mutagenesis was performed using the primer LMRC772 (5’-TGGCTGTTCAGCTCGCGTCGGAGGCCAGGCA GAA-3’), resulted in pMTS9. HA-tagged BMRF1 and BMRF1△TA expressing plasmids (pMT8 and pMT10) were generated by inserting the HA sequence into pYPW88 and pMTS9, respectively. The Rta-expression plasmid RTS15 and BALF5-expression plasmid pDH312 was gifts from Diane Hayward (Ragoczy et al. 1998) and Gao et al. (Gao et al. 1998).

The luciferase reporter plasmids containing promoter regions of BcLF1, BDLF3, BILF2 and BLLF1 were generated by PCR amplification and ligated into pGL2-basic vector as previously described (Chua et al. 2007). The pGL2-BLLF2 reporter plasmid was generated by ligation of the PCR products of the promoter region of BLLF2 (pBLLF2, nt 89951 to 91051 of the EBV B85.8 genome) using primers LMRC955 (5'-ATCGGTACCTTTCTGGTGCATTTGCGAGC-3’) and LMRC956 (5'-CTGAG ATCTCTGGTGGACACATGATGTGT-3'). pFastBac1, a Flag-BRG1 expression plasmid was a gift from Robert Kingston (Phelan et al. 1999).



5.2. Cell lines and transfection

HEK293T (293T) cell line is a derivative of a human kidney epithelial cell line (ATCC: CRL-1573). HeLa cell line was derived from human cervical epithelial cells (ATCC: CCL-2). NA cell, an EBV-positive cell line latently infected with recombinant Akata strain EBV(Chang et al. 1999), was selected from its parental cell, NPC-TW01, an EBV-negative nasopharyngeal carcinoma (NPC) epithelial cell line (Lin et al. 1990). The EBV lytic cycle can be induced in NA cells with the treatment of 40 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA) and 3 mM sodium butyrate (SB) (Chang et al. 1999). The 293TetER cell, an Rta-inducible cell line constructed from (T-REx^{TM}) 293 cell line (Invitrogen), carries inducible Flag-Rta plasmid (Chen et al. 2009). The Zta-inducible cell, 293TetEZ cell, was a generous gift from Dr. S.F. Lin (National Health Research Institutes, Taiwan). EREV8 cells, generated from the T-REx 293 cell line, carrie inducible Flag-Rta and also contain the Akata EBV genome. Doxycycline treatment of EREV8 cells induces Rta expression, which in turn triggers the EBV lytic cycle (Lee et al. 2008). DNA transfection was performed using Lipofectamine® 2000 transfection reagent (Invitrogen) according to the protocol suggested by the manufacturer. The cells were incubated for the indicated periods of time at 37℃ with 5% CO_2. Complementation of BKRF3 or BMRF1 plasmids into 293TetER/p2089BKRF3STOP or 293TetEZ/p2089△BMRF1 cell was performed with the calcium phosphate/ *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)-buffered solution (BBS) transfection protocol (Chen and Okayama 1987).

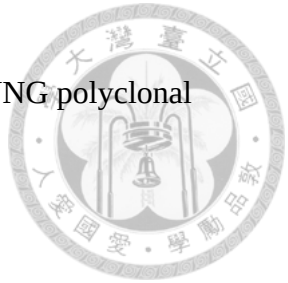
5.3. Immunofluorescence assay

Cells were cultured on fluorescence negative glass slides and transfected as required. At the harvest time points indicated, the slides were air dried and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 20 min. The slides were washed with PBS and incubated with anti-Flag (M2, Sigma-Aldrich), anti-HA (HA.11, Covance), anti-BRG1 (EPNCIR111A, Abcam) or rabbit anti-BALF5 serum (DP-1) at 37°C for 1.5 h, washed and subsequently incubated with a fluorophore-conjugated secondary antibody at 37°C for 1 h. Finally, the slides were stained with 100 ng/ml Hoechst 33258 at room temperature for 1 min and covered with H1000 mounting medium (Vector Labs), and observed by fluorescence (Zeiss, Axioskop 40 FL) or LSM 510 META confocal microscopy (Zeiss).

5.4. Western blot analysis

Western blotting analysis was performed as described previously (Chen et al. 2000). To detect the EBV lytic proteins, the primary antibodies used were lab-made mouse anti-BKRF3 serum #3, mouse anti-Zta 1B4, anti-Rta 467, anti-BGLF4 2616 and anti-BMRF 88A9 as described previously (Lee et al. 2007, Lu et al. 2007). The BALF2 antibody (OT13B) was kindly provided by Dr. Jaap M. Middeldorp (Zeng et al. 1997). Other primary antibodies used were anti-gp350/220 (72A1, ATCC), anti-HA antibody (HA.11, Covance), anti-Flag antibody (M2, Sigma), anti-BRG1 (EPNCIR111A,Abcam), anti-SP1 (PEP2, Santa Cruz Biotechnology), anti-PARP (F-2; Santa Cruz Biotechnology), anti-α-tubulin (DM1A, Calbiochem), anti-GAPDH (Biodesign),

anti-actin (Sigma-Aldrich), anti-myc (9E10) (Evan et al. 1985) and UNG polyclonal antibody (ab23926, Abcam).



5.5. Subcellular fractionation

The subcellular fractionation protocol was modified from a previous study (Lu et al. 2007). Briefly, trypsinized cells were harvested, washed and incubated with 1 ml hypotonic buffer (5 mM Tris-HCl pH 7.4, 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM dithiothreitol and 1 mM PMSF) with gentle shaking at 4℃ for 1 h. The cell suspension was then passed 15 times through a 26-gauge needle and centrifuged at 500 × *g* at 4℃ for 5 min. The pellet was collected as the nuclear fraction. The supernatant was mixed with 0.1 × volume of 72% trichloroacetic acid and 0.1 × volume of 0.15% deoxycholic acid with gentle shaking at 4℃ for 90 min and centrifuged at 15,000 × *g* at 4℃ for 20 min. The resulting pellet was washed with 95% EtOH twice and air dried, as the cytoplasmic fraction.

5.6. Co-immunoprecipitation assay

About 1×10^7 cells were lysed with 1 ml of CSK buffer (Lee et al. 2007) with gentle shaking at 4 ℃ for 2 h. Cell debris was precipitated by centrifugation at 16,000 × *g* at 4℃ for 10 min and supernatant was collected. The supernatant was pre-cleaned with 100 µl of 20% protein A-Sepharose beads (GE Healthcare) for 1 h at 4℃. The pre-cleaned lysate was then incubated with 1µg of anti-Flag, anti-Rta, anti-GST, anti-HA or anti-BRG1 antibody for 12 h at 4℃, followed by incubating with

100 µl of 20% protein A-Sepharose beads for 1 h at 4℃. Immunocomplexes were collected and washed with CSK buffer once and with cold PBS for three times. Immunocomplexes were disrupted by SDS-sample buffer, and the interacting proteins were detected by western blot analysis.

5.7. GST pull-down assay

Recombinant protein expression in *E. coli* transformants was induced by adding IPTG at the concentration of 0.1 mM to 100 ml of bacterial cultures of OD_{600} 0.6-0.8 and incubating at 25℃ for 2 h. The bacteria were then harvested and resuspended in 2 ml of PBST (1 × PBS, 1% Triton X-100 and 1 × protease inhibitor) with 1 mg/ml lysozyme, incubated on ice for 1 h and subjected to a -80 to 37℃ freeze-thaw cycle. The bacteria were disrupted with sonication on ice and the insoluble portion was removed by centrifugation at 12,000 × g for 10min. The buffer-soluble supernatant was mixed with 150 µl of 50% glutathione-Sepharose beads (GE Healthcare) and incubated on a rotating platform with a speed of 5 rpm at 4℃ for 2 h. The beads were washed three times with 1 ml of cold PBST, with 10 rpm rotation at 4℃ for 5 min. Cell lysates from mock, Rta-, or BALF5-transfected 293T cells were added and incubated with the beads in binding buffer (1% Triton X-100, 20 mM Tris-HCl pH 8.0, 140 mM NaCl, 1 mM EGTA, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM DTT, 10 µg/ml PMSF and 1 × protease inhibitor) at 4℃ for 18 h. The beads were then washed three times with 1 ml of washing buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl and 1% NP-40) and subjected to SDS-PAGE analysis.

5.8. Uracil-DNA glycosylase (UDG) assay

The single-stranded oligonucleotide LMRC-U (5'-AGCTACCATGCCTGCACGA AUTAAGCAATTCGTAATCATGGTCAT-3') was labeled with [γ-^{32}P]ATP at the 5' end, purified and quantified as previously described (Lu et al. 2004, Lu et al. 2007). Typically, labeled oligonucleotides equivalent to 4×10^5 cpm were incubated with *E. coli* UNG enzyme (NEB) or cell lysates at 37℃ for 10 min. A standard assay was carried out in 20 µl of the buffer containing 1 mM EDTA, 1 mM DTT, and 20 mM Tris-HCl, pH 8.0. The UDG activity was stopped by heating the reactions at 95℃ for 5 min. After glycosylase cleavage, abasic sites were incised by 0.1 mM NaOH treatment at 95℃ for 5 min. Reaction products were analyzed with electrophoresis on 15% (wt/vol) polyacrylamide denaturing gels (7 M urea, 1 × Tris-borate-EDTA), and the gels were dried and subjected to autoradiography. The cleavage percentage of U-probe was quantified with ImageQuant (GE Healthcare).

5.9. Construction of the BKRF3 knockout and revertant EBV bacmids

The shuttle vector pGS284, donor strain S17λpir *E.coli* (GS111) and recipient strain GS500 (*recA*$^+$) used for EBV allelic exchange, were generous gifts from Dr. R. Sun (University of California, Los Angeles) (Jia et al. 2004). The 5'- and 3'- flanking region of BKRF3 (B95.8 strain 110072-111072) was amplified by PCR using primers: LMRC785 (5'-GAAGATCTCTTCTCGCGTTGGAAAACATTAGCGAC-3'), and LMRC786 (5'- AAGATCTTTAGCGAGGACAAAGTGGTTGTTGCCC-3'). The PCR product was digested, and cloned into the BglII site of pGS284, to generate pIH1

(pGS284/BKRF2-3). Sequentially, the stop cassette containing triple-ORF nonsense codons and an NheI site was inserted into pIH1 between 110572 and 110573 of the B95.8 genome by double-primer PCR mutagenesis (Edelheit et al. 2009) using primers: LMRC808 (5'-CATGTGCATGGCCGCTAGCTTGATTAATTGATGGGCCCGCTTT T-3') and LMRC809 (5'-AAAAGCGGGCCCATCAATTAATCAAGCTAGCGGCC ATGCACATG-3'). The resulting plasmid, pIH3 (pGS284/BKRF3STOP), was electroporated into E.coli strain GS111 for allelic exchange. The EBV bacmid p2089 (Delecluse et al. 1998), a kind gift from H. J. Delecluse (DKFZ unit F100, Heidelberg, Germany), was electroporated into E.coli strain GS500 (*recA*$^{+}$). For allelic exchange, conjugation was performed by cross-streaking the GS500/p2089 and GS111/pIH3 on LB agar at 37°C for 16 h, and recombinant bacmids were selected according to a previously described procedure (Smith and Enquist 2000). The incorporation of the stop codon in BKRF3 open reading frame was determined by colony PCR using primers LMRC785 and 786, and restriction enzyme digestion for the insertion of the NheI site. Furthermore, the GS500/p2089BKRF3STOP bacmid was cross-streaked with GS111/pIH1 to generate the revertant bacmid of p2089BKRF3STOP by allelic exchange.

5.10. Construction of the BMRF1 knockout EBV bacmid

The BMRF1 knockout bacmid was constructed by PCR targeting as described (Datsenko and Wanner 2000). In brief, the apramycin resistant cassette was amplified by PCR from pIJ773 using primers LMRC 718 (5'-TCTGCTCTGGTACGTTGGCTT

CTGCTGCTGCTTGTGATCTGTAGGCTGGAGCTGCTTC-3') and LMRC 719 (5'-C CGTACTGGCGGCCGCCTCTTCGGAGGCGTGGTTAAATAATTCCGGGGATCCG TCGACC-3'). The apramycin gene products were transformed into E.coli DH10B containing Maxi-EBV bacmid p2089 and Red recombinase plasmid pKD46 by electroporation. The recombination of PCR products with wild-type EBV genome resulted in the exchange between the BMRF1 gene region 79899-808778 of EBV B95.8 and apramycin cassette. The p2089△BMRF1 bacmid was selected by apramycin and confirmed by BamHI digestion.

5.11. Selection of doxycycline inducible cells containing EBV bacmid DNA

To select doxycycline inducible EBV bacmid-positive cell lines, 293TetER or 293TetEZ cells (5×10^5 cells/well) were seeded in 6-well culture plate and transfected with 7 μg of EBV p2089 wild-type or mutant bcmid using T-Pro NTRII transfection reagent (T-Pro Biotechnology). At 72 h posttransfection, transfected cells were split into two 10-cm culture dishes and selected with hygromycin B (100 μg/ml) for one month. Four to 6 GFP-positive cell colonies were picked up to obtain pool clones. More than 10 pooled clones of individual transfected cells were selected. The selected EBV wild-type or mutant clones were treated with doxycycline (50 ng/ml) to confirm successful lytic induction by western blotting analysis.

5.12. Immunoprecipitation-UDG assays

Cells were harvested and resuspended in extraction buffer (10 mM Tris-HCl, pH

8.0, 200 mM KCl, 1mM EDTA, 20% glycerol, 0.25% NP-40, and 1 mM DTT, 1 × protease inhibitor). The cell mixture was rotated at 4°C for 3 h and cell debris was removed by centrifugation at 16000 × g for 5 min. Two hundred micrograms of cell extract were incubated with 1 μg anti-HA antibody on a rotating shaker at 3 rpm at 4°C for 3 h. Subsequently, 100 μl of 20% protein A-Sepharose beads were added into the mixture and incubated at 4°C for 1 h. Immunocomplexes were collected and washed three times with 10 mM Tris-HCl (pH 7.4) at 4°C for 15 min. The 10% of protein A beads bound immunocomplexes were resuspended in 2 μl UDG extraction buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 250mM NaCl, and 1 mM DTT) for UDG assay; the 90% of immunocomplexes were analyzed by western blotting.

5.13. Genomic DNA extraction and quantitative real-time PCR for EBV copy number

Cells were lysed in 400 μl digestion buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% SDS, 0.1 mg/ml proteinase K) and incubated at 55°C for 3 h. RNase A (0.5 mg/ml) was then added and incubated at 55°C for another 20 h. The genomic DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated by adding 2 volumes of 100% ethanol and 1⁄10 volume of 3 M sodium acetate (pH 5.2). Twenty-five ng of total DNA were evaluated for EBV genomic DNA by PCR or Q-PCR using following BamHI W primers: 5’-CCCTGGTATAAAGTGGTCCT-3’ and 5’-AAGTCCACTTACCTCTGG-3’. The copy number of cellular β-globin was detected for normalization using following

primers: 5'-GGTTGGCCAATCTACTCCCAGG-3' and 5'-GCTCACTCAGTGGCA AAG-3'. For Q-PCR, EBV DNA was quantified using SensiFAST SYBR® No-ROX Kit (Bioline). The standard curve for Q-PCR was generated by a 10-fold serial dilution of a mixture of 10^4 copies of genomic DNA of 293TetER cells and 5×10^6 copies of purified EBV bacmid DNA.

5.14. DNA binding assay with single-stranded DNA cellulose

The [^{35}S]methionine labeled wild-type and mutant HA-BKRF3 proteins prepared by in vitro TNT® Quick Coupled Transcription/Translation Systems (Promega) were used for ssDNA cellulose chromatography. The single-stranded calf thymus genomic DNA cellulose resin (Sigma-Aldrich) was equilibrated with the binding buffer (50 mM Tris-HCl, pH 8.0, 1 mM PMSF and 10% glycerol). Thirty μl of in vitro transcription/translation products were diluted 1:10 in binding buffer containing 100 μg RNase A and applied to a 200 μl bed volume of ssDNA cellulose resin in a Poly-Prep chromatography column (Bio-Rad). After washing with 300 μl binding buffer, the bound proteins were eluted with 300 μl serial gradients of binding buffer containing 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl. The 3 μl of transcription/translation products and 30 μl of eluents were subjected to electrophoresis in 15% SDS-PAGE gels and analyzed with a Typhoon Trio Variable-Mode Imager (GE Healthcare).

5.15. EBV DNA Microarray analysis

Twenty micrograms of total RNA from 293TetER(Z)/p2089 wild-type or 293TetER(Z)/p2089△BMRF1 cells were reverse transcribed and labeled with

biotin-dUTP as described (Chen et al. 1998). Hybridization of biotin-labeled cDNA with EBV DNA microarray was performed following the protocol described previously (Lu et al. 2006). In brief, the microarray membrane was pre-hybridized with salmon sperm DNA at 60 °C for 90 min. After pre-hybridization, microarray membrane was incubated with biotin-labeled cDNA at 63 °C for 15 h. Following hybridization of biotin-labeled cDNA probe, microarray membrane was washed with 2x SSC washing buffer (0.3 M NaCl, 30 mM sodium citrate, 0.1% SDS) and the membrane was subjected for color development. The microarray chromogenic image was scanned and converted to digital output by ImageJ 1.48.

5.16. RT-PCR and RT-qPCR

Cells were harvested for RNA purification using TRIzol® Reagent (Life technologies). Before reverse transcription, 10 μgs total RNA samples were pre-treated with 10 U of DNase I (Life technologies). Subsequently, RNA was reverse transcribed into cDNA for viral gene detection by PCR or qPCR as described (Lu et al. 2006). The primers used for detection of viral transcripts were listed Table 4. The β-actin and GAPDH transcripts were detected as internal control using primers β-actin forward (5'-TTCTACAATGAGCTGCGTGT-3'), β-actin reverse (5'-GCCAGACAGCACTGTG TTGG-3'), GAPDH forward (5'-CATCATCCCTGCCTCTACTG-3'), and GAPDH reverse (5'-GCCTGCTTCACCACCTTC-3').

5.17. Luciferase assay

293T cells were transfected with pBLLF1-luc, pBLLF2-luc, pBcLF1-luc, pBDLF3-luc or pBILF2-luc, the amounts of effector plasmids indicated and Renilla luciferase control reporter vector (pRL-null) as a control for transfection efficiency. At 48 h posttransfection, cells were harvested and assayed for firefly and Renilla luciferase activities by using the Dual-Glo Luciferase Assay system (Promega). Promoter activities were determined from firefly luciferase activity and normalized with Renilla luciferase activity in each reaction. Relative fold activation indicates the ratio of reporter activity to that of vector-transfected cells.

5.18. Immunoprecipitation -spectrometry assay

For immunoprecipitation of BMRF1 associated complex, 293TetEZ/p2089△BMRF1 cells were transfected with a Flag-tagged BMRF1 and induced into lytic cycle progression for 48h. After lytic induction, the cells were lysed by NP40-lysis buffer (50 mM Tris HCl, pH 8.0, with 150 mM NaCl, 2 mM EDTA, 1% NP40 and 1 × protease inhibitor), and Flag-BMRF1was precipitated by anti-Flag M2 antibody (Sigma Aldrich). The BMRF1-containing complexes were captured by protein A-Sepharose beads (GE Healthcare) and the captured complexes were analyzed by SDS-PAGE and silver staining. The indicated bands were excised and trypsin digested. Resulting peptide fragments were extracted from the gel to perform mass spectrometry at proteomics and protein function core laboratory (Center of Genomic Medicine, National Taiwan University) . The BMRF1-interacting proteins were identified via

peptide mass fingerprint, searching all entries in the National Center for Biotechnology Information and SwissProt databases.

5.19. shRNA lentivirus production

Short hairpin RNA (shRNA) plasmids pLKO.1-SP1, pLKO.1-BRG1 and pLKO.1-Luc were obtained from the National RNAi Core Facility (Academia Sinica, Taiwan; http://rnai.genmed.sinica.edu.tw). The shRNA target sequences for SP1 is CCAGGTGCAAACCAACAGATT (TRCN00000020447). The shRNA target sequences for BRG1 are CTTTGCGTATCGCGGCTTTAA (TRCN0000231101), CGGCAGACACTGTGATCATTT (TRCN0000015552) and CCCGTGGACTTCAAG AAGATA (TRCN0000015549). The pLKO.1-shLuc plasmid is a non-targeting shRNA control. For lentiviral packaging, 293T packing cell were seeded and transfected with shRNA-pLKO.1, pCMV-dR8.91 (Delta 8.9) and pMD.G plasmids in the ratio 10:9:1 by BBS transfection. The transfected cells were incubated at 30°C and 3% CO_2. At 18 h posttransfection, cells were replaced with fresh medium supplemented with 10% FBS and 1% penicillin/streptomycin and 1% BSA. The culture supernatants containing lentiviruses were harvested at 36 and 48 h after transfection.

Tables and Figures

Table 1. Viral proteins acting at EBV oriLyt and their functions

Protein	Function	Reference
BZLF1 (Zta)	1. Origin-binding protein of oriLyt that can bind with multiple viral replication proteins	(Fixman et al. 1995, Schepers et al. 1996)
	2. Transcription factor required to initiate the lytic phase of EBV and to activate the expression of viral proteins of lytic DNA amplification	(Farrell et al. 1989, Feederle et al. 2000)
BRLF1 (Rta)	Transcription factor required to initiate the lytic phase of EBV in epithelial cell and to activate a number of viral early and late gene expression	(Ragoczy and Miller 1999)
BALF5	DNA polymerase	(Lin et al. 1991)
BALF2	Single-stranded DNA-binding protein	(Tsurumi et al. 1996)
BMRF1	1. DNA polymerase processivity factor	(Li et al. 1987)
	2. Transcription factor require to turn on BHLF1 promoter	(Zhang et al. 1996)
	3. Transcriptional coactivator of BZLF1	(Nakayama et al. 2009)
BBLF4	DNA helicase BBLF4, BSLF1 and BBLF2/3 form the helicase-primase complex	(Yokoyama et al. 1999)
BSLF1	Primase	
BBLF2/3	Primase-accessory protein	

Table 2. The regulation fold of BMRF1-mediated EBV genes in Rta or Zta inducible cells

ORF	Putative Function	293TetER △BMRF1/WT	293TetEZ △BMRF1/WT
BERF4	EBNA 3C	-14.829	-8.216
BcLF1	Major capsid protein	-11.007	-2.030
BLLF1b	Glycoprotein gp350/220	-9.246	-3.646
BFLF2	nuclear egress protein	-8.659	ND
BDLF2	Cycline B homolog	-8.413	0.980
BaRF1	Ribonucleotide reductase	-7.304	-4.893
BFLF1	Glycoprotein	-6.755	-2.117
BYRF1-1	EBNA2	-6.580	ND
BXLF2	Glycoprotein H	-6.340	0.428
BFRF3	Capsid protein	-5.956	-2.069
BVRF2	Capsid protein	-5.925	-1.107
BERF1	EBNA3A	-5.428	0.239
BILF2	Glycoprotein gp78/55	-3.801	-2.497
BZLF2	Glycoprotein gp42	-3.613	ND
BNLF1	LMP1	-3.419	ND
BLLF2		-3.147	-3.769
BLLF1 5'	Glycoprotein gp350/220	-3.032	-6.699
BLRF3	EBNA3A	-2.527	1.962
BORF2	Ribonucleotide reductase	-2.370	-4.492
BHRF1	viral bcl-2	-2.271	-3.985
BILF1	Glycoprotein gp60	-2.195	-3.548
BGRF1	Packaging protein	-2.086	2.647
BFRF2		-2.036	-1.840
BTRF1		-2.028	-5.489
BCRF2		-1.858	-0.928
BDLF3	Glycoprotein gp150	-1.814	-3.470

ND, non determine

Table 3. The BMRF1-associated proteins analyzed by Mass-spectrometry

BMRF1 interacting candidate proteins increased in lytic cycle	No. of peptides by MS
Replication/repair/recombination	
DNA-dependent protein kinase catalytic subunit (DNA-PKcs)	8
Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD)	14
DNA topoisomerase 2-alpha (TOP2A)	2
DNA topoisomerase 2-beta (TOP2B)	2
Chromatin remodeling	
Chromodomain-helicase-DNA-binding protein 4 (CHD4)	11
SWI/SNF-Related Matrix-Associated Actin-Dependent Regulator of Chromatin Subfamily A Member 4 (SMARCA4, BRG1)	6
Structural maintenance of chromosomes flexible hinge domain-containing protein 1 (SMCHD)	6
AT-rich interactive domain-containing protein 4B (ARID4B)	3
Retinoblastoma binding protein 4 (RBBP4)	4
RNA binding/splicing	2
U5 small nuclear ribonucleoprotein 200 kDa helicase (SNRNP200)	7
Polypyrimidine tract-binding protein 1 (PTBP1)	4
Other	
Nuclear mitotic apparatus protein 1 (NUMA1)	2
Vimentin	3
BMRF1 interacting candidate proteins decreased in lytic cycle	**No. of peptides by MS**
Chromatin remodeling	
Histone deacetylase 2 (HDAC2)	6
RNA binding/splicing	
Heterogeneous nuclear ribonucleoprotein K (HNRNPK)	7
U4/U6 small nuclear ribonucleoprotein Prp31	6
Protein folding	
T-complex protein 1 subunit theta (CCT8)	3

Mass spectrometry analysis was performed at Center of Genomic Medicine, National Taiwan University

Table 4. EBV primers used for RT-PCR and RT-qPCR

ORF	Forward primer sequence	Reverse primer sequence
BcLF1	5'-GGTCGTGTACTTGGGATTG-3'	5'-CCCCATCTCCCTCTTACC-3'
BLLF1	5'-TACTGGGGTGGGACTTGTT-3'	5'-GAGCGGGGGAGATTACTG-3'
BLLF2	5’-TGACCAGCAGCAGCAGAAG-3’	5’-ATGTGTCCACCAGTTCGCC-3’
BlLF2	5’-ACTGGTGCTTGTGGTGTG-3’	5’-CTGTGTGGGGTCTGTTTGT-3’
BSRF1	5’-GGCGGGCTAAACAGAACGA-3’	5’-AAGGCGGAGTTGATGAAAG-3’
BdRF1	5’-CAGACCCCGCTACCCTAC-3’	5’-GGTGGGCTCCTTAGACTG-3’
BaRF1	5’-TCCTTCTACAGCATAGCCCT-3’	5’-AGGTCATCTACCACCAGCAT-3’
BORF2	5’-GGACACCCACCACACAGCA-3’	5’-TCAAACTCCTCCCCGTAGA-3’

Fig. 1. Functional domains of BMRF1.

The amino acid 1 to 303 of BMRF1 is critical for its DNA binding ability, and is essential for DNA polymerase accessory function. The carboxyl-terminus of BMRF1 contains the transactivation domain and nuclear localization signal (NLS).

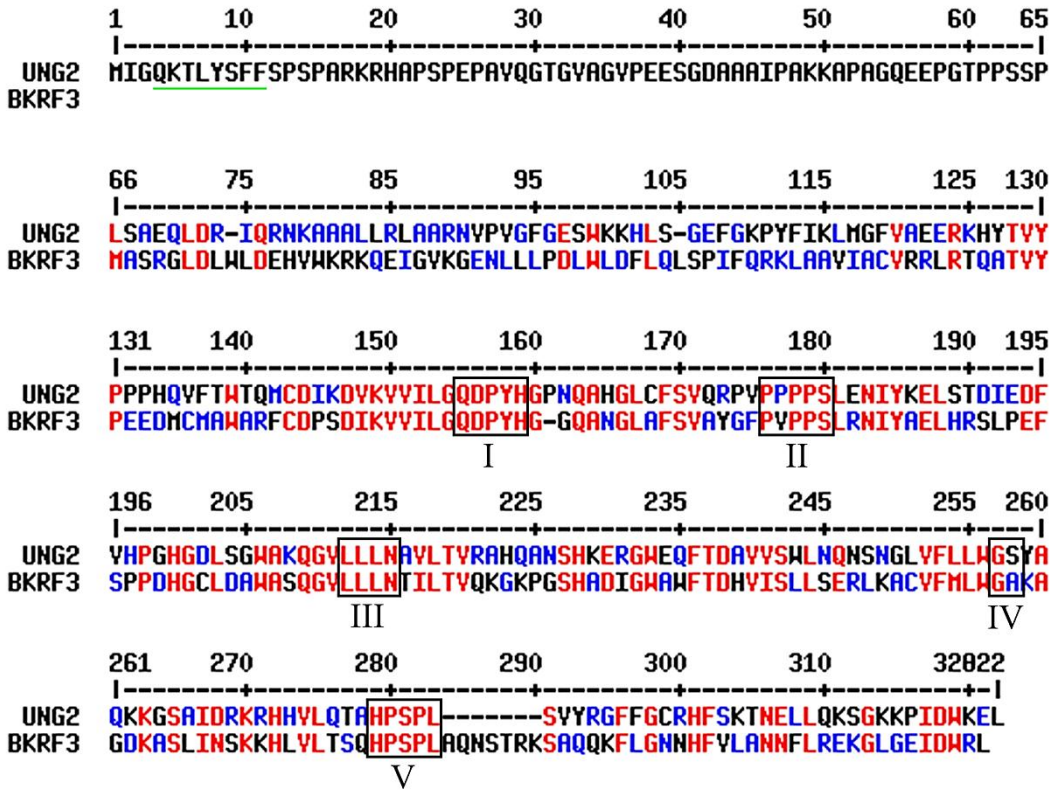
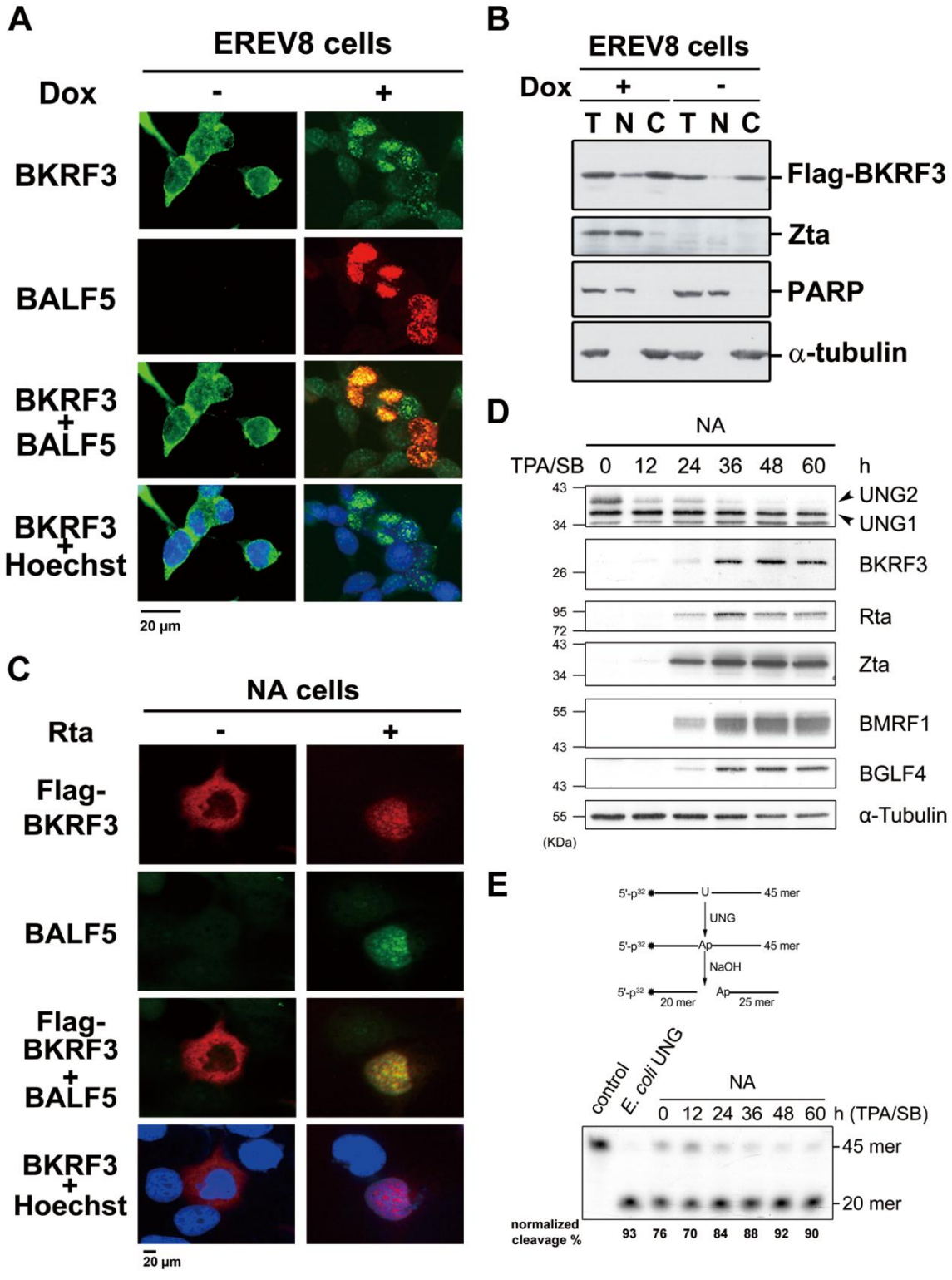


Fig. 2. Sequence alignment of human UNG2 and EBV BKRF3.

The protein sequence of BKRF3 is aligned with human UNG2 using MultAlin software (Corpet 1988). The high consensus residues are indicated in red, and the low consensus residues are indicated in blue. Boxed residues indicate functional motifs involved in DNA binding and enzymatic activity: (I) the water activating loop; (II) the 4-Pro loop; (III) the uracil recognition β2-strand; (IV) the GS-loop; (V) the leucine loop. The nuclear localization signal (NLS) of human UNG2 is underlined in green.

Fig. 3. Translocalization of BKRF3 during the lytic cycle.

(A) In EREV8 cells, an Rta-inducible EBV-positive cell line, BKRF3 protein was mostly distributed in the cytoplasm before Rta expression is induced (left) and is transported into the nucleus and colocalized with BALF5 DNA polymerase after induction with 100 ng/ml doxycycline for 48 h (right). (B) Western blotting of fractionations of cell lysates indicates that in noninduced EREV8 cells, BKRF3 was mainly in the cytoplasmic (C) fraction, whereas after induction, a greater amount of BKRF3 was also detected in the nuclear (N) fraction. T, total; PARP, poly-ADP-ribose polymerase, a nuclear protein marker. α-Tubulin serves as a cytoplasmic marker. (C) In EBV-positive NA cells cotransfected with vector control, Flag-BKRF3 was distributed in the cytoplasm at 48 h posttransfection (left) and was translocated to the nucleus when viral replication was induced by cotransfection of the Rta expression plasmid (right). The BKRF3 protein may interact with the viral DNA replication-associated proteins, as evidenced by its colocalization with BALF5 DNA polymerase in cells with virus replication. (D) To monitor temporal expression of EBV BKRF3 and cellular UNGs, lytic replication was induced in EBV-positive NA cells with TPA-SB. BKRF3 expression was detected at 12 h and significantly upregulated at 36 h postinduction. The protein expression levels of mitochondrial UNG1 remained stable, and nuclear UNG2 decreased through the time course. (E) The upregulated BKRF3 may compensate for the reduced amount of cellular UNG2 according to the results of the UDG activity analysis. (Experiments A and B were performed by Su-Ming Chang; Experiments D and E were performed by I-Hwa Liu)


A
EREV8 cells
Dox
-
+
BKRF3
BALF5
BKRF3 + BALF5
BKRF3 + Hoechst
20 μm
B
EREV8 cells
Dox
+
-
T N C T N C
Flag-BKRF3
Zta
PARP
α-tubulin
C
NA cells
Rta
-
+
Flag-BKRF3
BALF5
Flag-BKRF3 + BALF5
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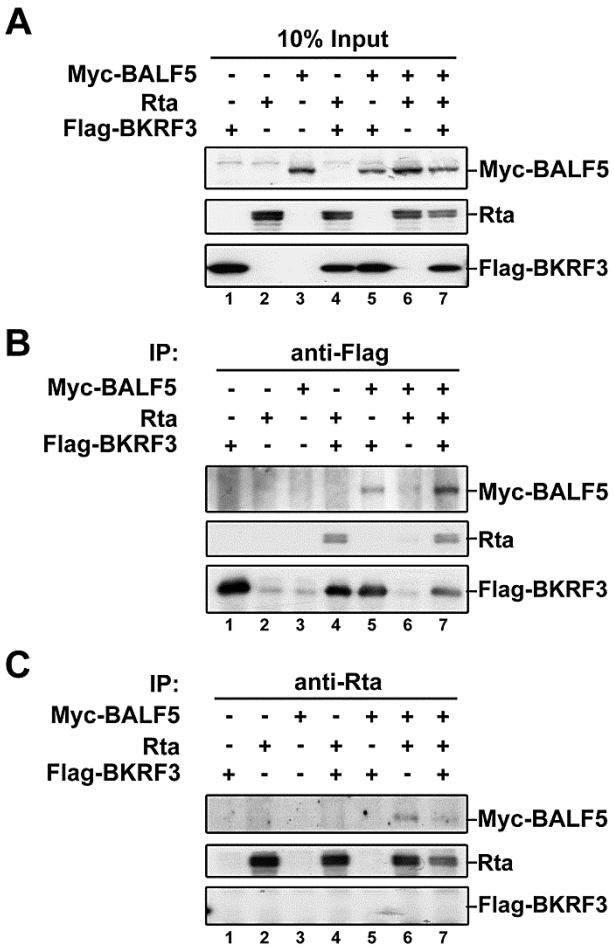


Fig. 4. BKRF3 interacts with viral DNA replication-associated proteins.

Immunoprecipitation (IP) and Western blotting assays were used to examine protein interactions among BKRF3, BALF5, and Rta in a transient cotransfection system. (A) Transfection of plasmid DNA expressing BALF5, Rta, or BKRF3 into 293T cells shows the expression of each protein. Vector plasmids were supplemented to ensure equal amounts of total DNA in each reaction. Cell lysates of transfected cells were displayed by Western blotting and immunoprecipitated with anti-Flag (B) or anti-Rta (C) and detected with anti-BALF5, anti-Rta, or anti-Flag in the subsequent Western blotting. (Experiments A to C were performed by Su-Ming Chang)

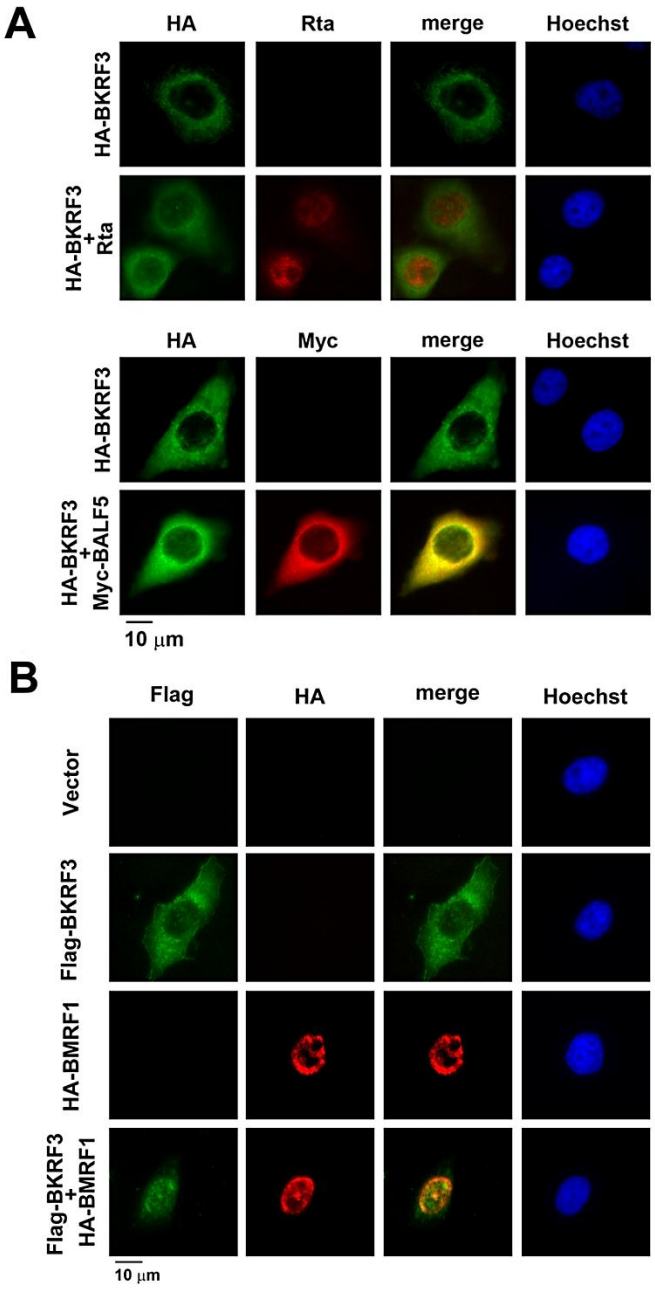


Fig. 5. BKRF3 is translocated into the nucleus in the presence of BMRF1.

(A) HA-BKRF3 plasmid was transfected with vector control, Rta, or Myc-BALF5 plasmid into HeLa cells and detected with anti-HA, anti-Rta (467), or anti-Myc (9E10) antibody at 24 h posttransfection in an immunofluorescence assay. DNA was stained with Hoechst 33258. (B) Flag-BKRF3 was transfected into HeLa cells with HA-BMRF1 or control plasmid. At 24 h posttransfection, cells were fixed and stained for BKRF3, BMRF1, and DNA with anti-Flag antibody, anti-HA antibody, and Hoechst 33258, respectively.

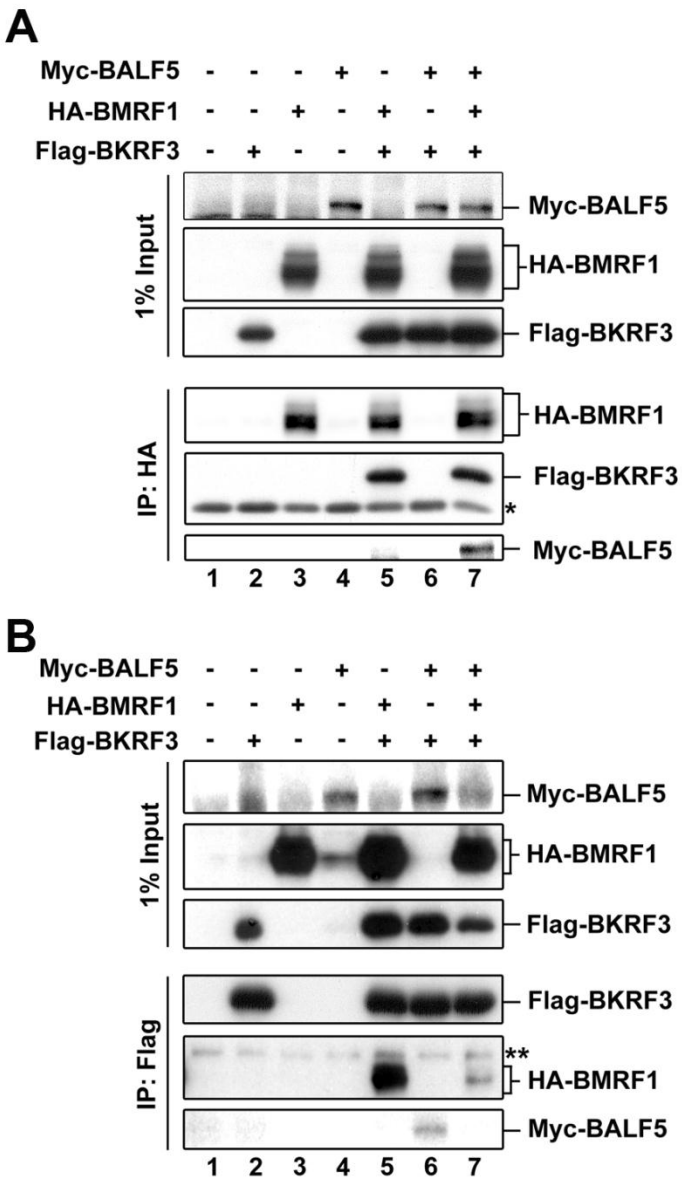


Fig. 6. BKRF3 interacts with viral replication-associated proteins, including BALF5 and BMRF1.

293T cells were transfected with the plasmids indicated for 24 h and harvested using radioimmunoprecipitation assay (RIPA) buffer. Cell lysates were incubated with anti-HA (A) or anti-Flag (B) antibody to precipitate BMRF1-associated or BKRF3-associated immunocomplexes and examined for BALF5, BMRF1, or BKRF3 by Western blotting. *, Ig light chain; **, Ig heavy chain.

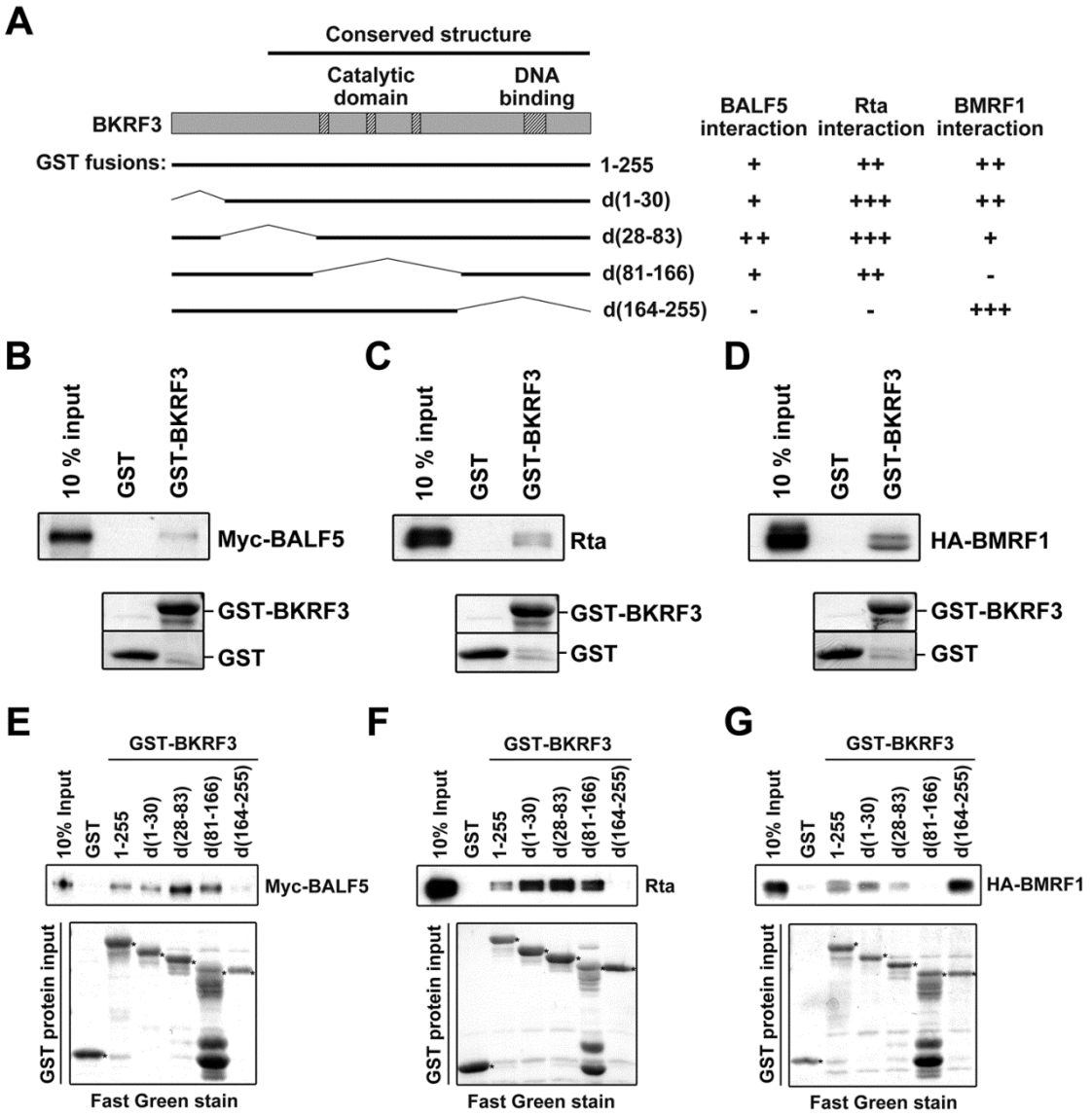


Fig. 7. Mapping the interaction domains of BKRF3 using a GST pulldown assay.

(A) Four GST-fused deletion mutants of BKRF3 were constructed, d(1-30), d(28-83), d(81-166), and d(164-255). The relative BKRF3-interacting abilities of individual GST fusion proteins are summarized on the right. (B to G) Bacterially expressed GST or GST-fused BKRF3 proteins were purified with glutathione beads and incubated with lysates of 293T cells transfected with Myc-BALF5, Rta, or HA-BMRF1 for 18 h. The beads were washed and examined with anti-Myc (B and E), anti-Rta (C and F), and anti-HA (D and G) antibodies by Western blotting.

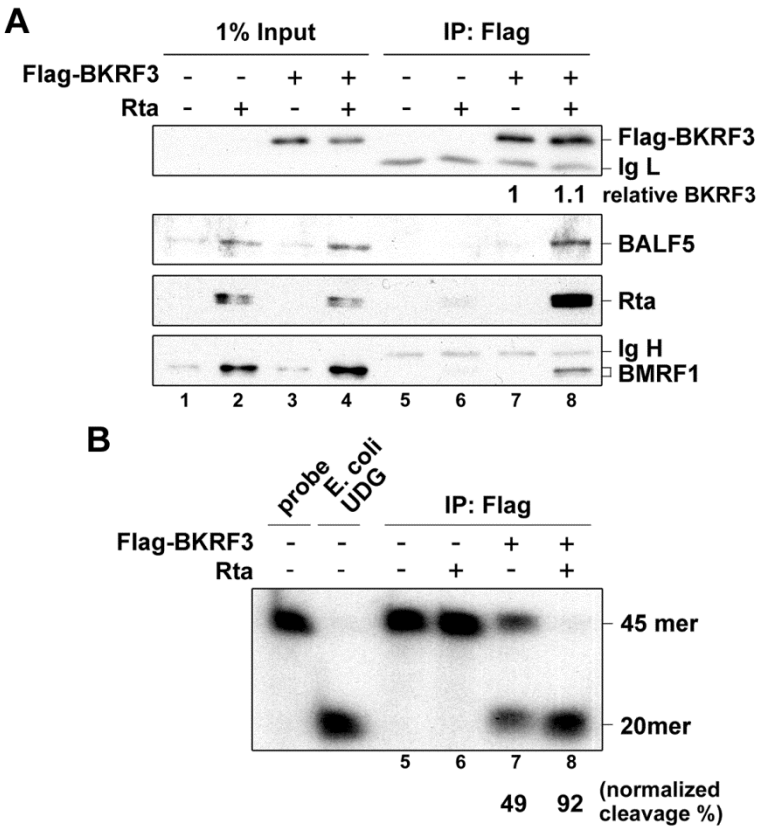
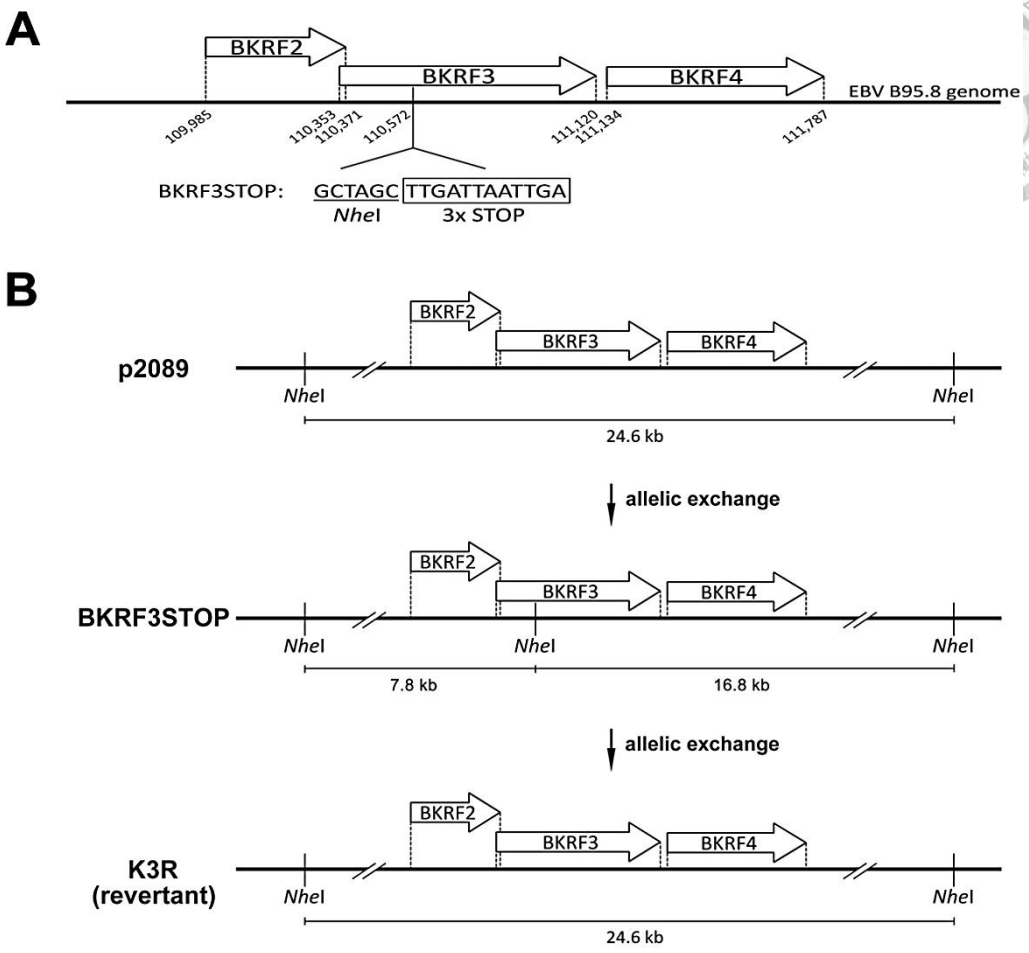


Fig. 8. Complex formation with viral DNA replication machinery in Rta reactivated NA cells stimulates BKRF3 UDG activity.

NA cells were seeded and transfected with vector or BKRF3-expressing plasmid, coupled with Rta or uncoupled, to induce lytic replication. At 60 h posttransfection, whole-cell lysates were harvested and Flag-BKRF3 was immunoprecipitated by anti-Flag (M2) antibody for coimmunoprecipitation assay and IP-UDG assay. (A) Ninety percent of Flag-BKRF3-associated immunocomplexes were examined for viral lytic proteins, including Rta, BALF5, and BMRF1 by immunoblotting assay. (B) Ten percent bed volumes of immunoprecipitated products were subjected to IP-UDG assay. The percentage of U-probe cleavage was quantified by ImageQuant (GE Healthcare) and normalized to the relative fold of immunoprecipitated Flag-BKRF3, as shown in panel A. Data are representative of two independent experiments.

Fig. 9. Construction and characterization of BKRF3 knockout and the revertant EBV bacmids.

(A) Schematic summary of the BKRF3 mutant EBV bacmid cloning strategy. The termination cassette containing an NheI site and translation stop codons was inserted into nucleotide 110572 of the EBV B95.8 genome (GenBank accession no. V01555.2) by allelic exchange, as described in Materials and Methods. (B) Recombination of allelic exchange led to a size change of NheI fragments from 24.6 kb to 16.8 and 7.8 kb, resulting in the disruption of BKRF3 without interfering with the coding sequence of BKRF2. The BKRF3 revertant bacmid (K3R), which contains the same genomic pattern with which the wild-type bacmid was generated, used BKRF3STOP bacmid as the backbone. (C) The wild-type, BKRF3STOP, and K3R bacmids were digested with BamHI or NheI and displayed by agarose gel electrophoresis. The fragmented viral DNA of wild-type, BKRF3STOP, and K3R bacmids were identical in BamHI digestion (left). In addition, cleavage of the BKRF3STOP bacmid produced 16.8- and 7.8-kb fragments (right, lane 2) that replaced the wild-type 24.6-kb fragment (right, lane 1), and the NheI-STOP cassette was replaced with the wild-type genome by allelic exchange-generated K3R (right, lane 3).


A
BKRF2
BKRF3
BKRF4
EBV B95.8 genome
109,985
110,353
110,371
110,572
111,120
111,134
111,787
BKRF3STOP: GCTAGC TTGATTAATTGA
NheI
3x STOP
B
p2089
BKRF2
BKRF3
BKRF4
NheI
NheI
24.6 kb
allelic exchange
BKRF3STOP
NheI
NheI
NheI
7.8 kb
16.8 kb
allelic exchange
K3R
(revertant)
24.6 kb
C
kb
p2089
BKRF3STOP
K3R
10
8
6
5
4
3
1
2
3
BamHI
12
10
8
6
5
4
24.6
16.8
7.8
NheI

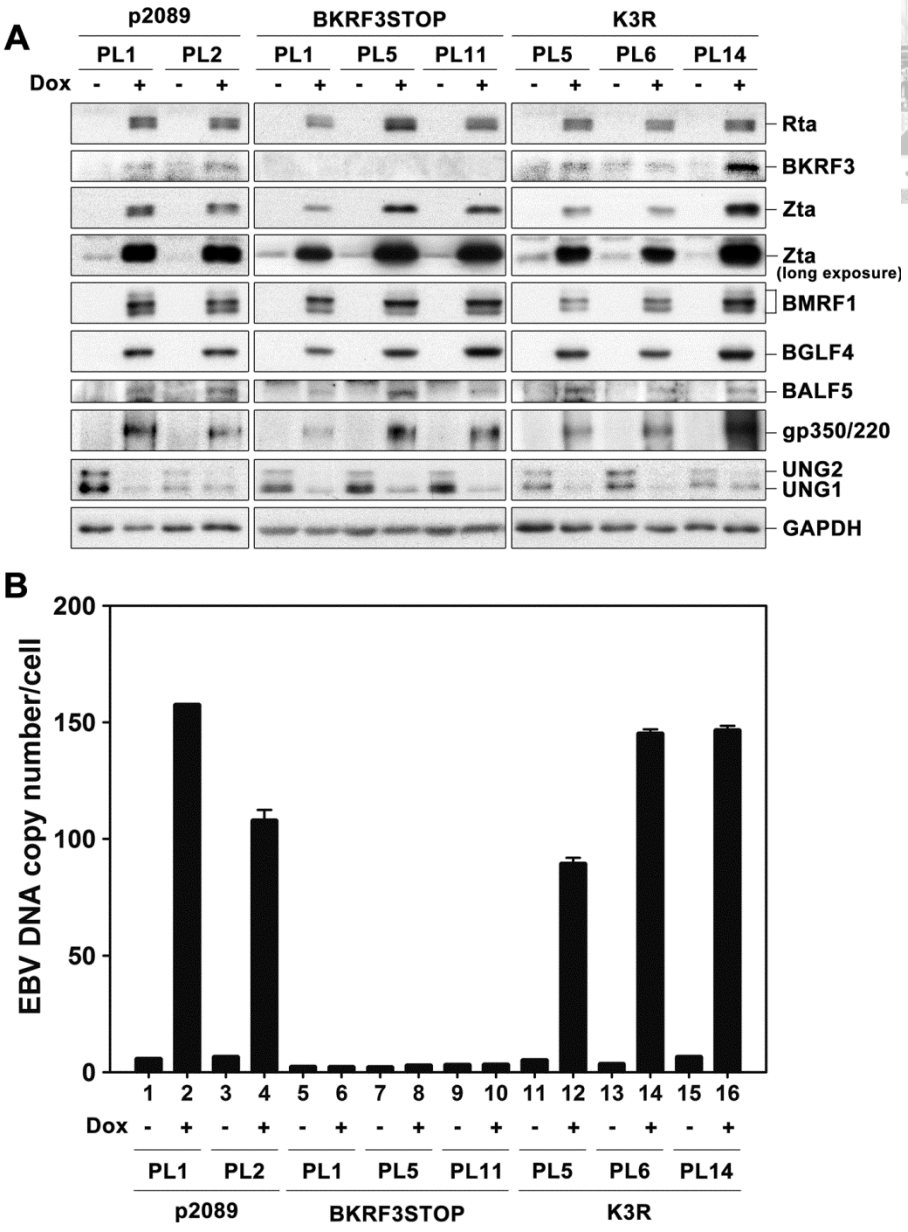
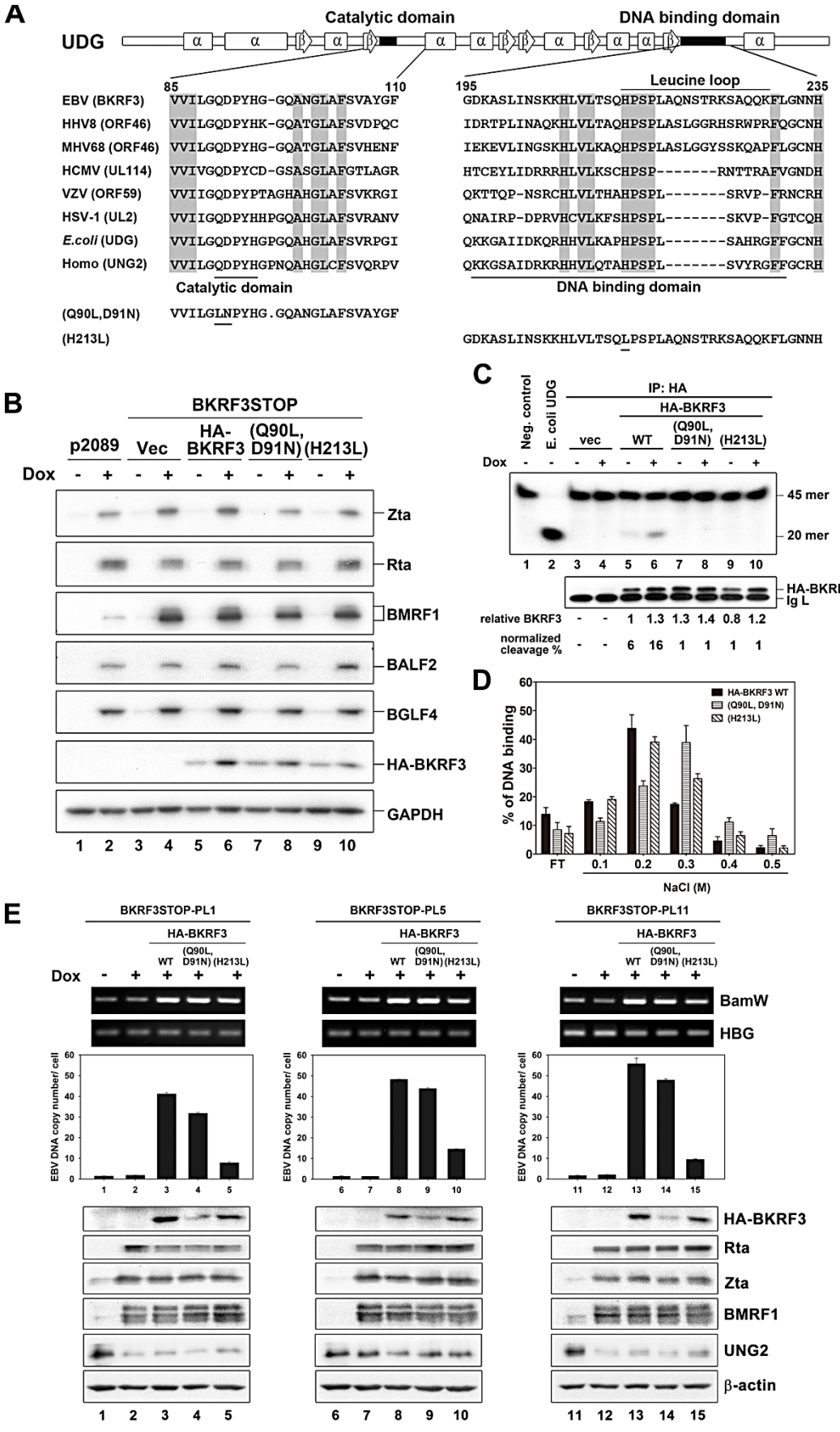


Fig. 10. EBV genome replication was deficient in cells containing BKRF3 knockout bacmids.

(A) Wild-type p2089, BKRF3STOP, and K3R EBV bacmids were transfected into 293TetER cells, and stable clones were selected with hygromycin (50 μg/ml). EBV bacmid stable cells (p2089, BKRF3STOP, and K3R) weretreated with doxycycline (50 ng/ml) to induce the EBV lytic cycle for 72 h, and viral lytic proteins were detected by immunoblotting. (B) The EBV genome copy number of wild-type p2089, BKRF3STOP, and K3R cells with or without doxycycline induction was determined by qPCR, using β-globin as an internal control, and results were compared to those for a 10-fold serial dilution of standard DNA as described in Materials and Methods.

Fig. 11. Trans-complementation by wild-type or enzymatically dead BKRF3(Q90L,D91N), but not leucine loop mutant BKRF3(H213L), bacmids rescued lytic viral DNA replication in BKRF3STOP cells.

(A) Sequence alignment of the catalytic domain and DNA binding domain of UDG within the herpesviruses, E. coli, and humans. Residues conserved within UNG (family 1) are marked in gray boxes. The domains of catalytic activity and DNA binding are indicated at the bottom, and the leucine loop involved in the DNA binding domain is indicated at the top. Secondary structures of EBVUDGare illustrated above the alignment. The mutation sites of enzymatically dead BKRF3(Q90L,D91N) and leucine loop mutant BKRF3(H213L) are indicated. (B) p2089 and BKRF3STOP cells were transfected with 7, 14, or 4 μg of HA-tagged wild-type BKRF3 (WT), BKRF3(Q90L,D91N), or BKRF3(H213L) plasmid, respectively, and treated with doxycycline (50 ng/ml) at 12 h posttransfection. After Doxycycline (Dox) treatment for 60 h, cells were harvested for Western blotting of viral lytic proteins. (C) The UDG activities of HA-tagged BKRF3 WT, BKRF3(Q90L,D91N), and BKRF3(H213L) in BKRF3STOP cells treated with doxycycline or left untreated. The transfected cell lysates were prepared for IP-UDG assays as described in Materials and Methods. *E. coli* UDG (10 U) was used as a positive control. The cleavage percentage of input 32P-labeled U probe was normalized to relative amounts of immunoprecipitated BKRF3. (D) The DNA binding activities of *in vitro*- transcribed/translated [35S]methionine-labeled BKRF3 WT, BKRF3(Q90L,D91N), and BKRF3(H213L) were measured in the binding buffer with ssDNA cellulose chromatography, eluted with binding buffer with step gradients of NaCl, and subjected to electrophoresis with 15% SDS-PAGE. The DNA-bound [35S]methionine-labeled proteins in each eluent were measured by phosphorimaging and normalized to the total input. Data are representative of two independent experiments. (E) BKRF3STOP pool clones (PL1, PL5, and PL11) were transfected with control vector (vec), HA-BKRF3 WT, BKRF3(Q90L,D91N), or BKRF3(H213L). At 24 h posttransfection, doxycycline (Dox; 50 ng/ml) was added to induce lytic replication for 60 h. Subsequently, intracellular EBVDNAcopy numbers of *trans*-complementation cells were quantified with qPCR for the EBV BamHI W fragment and human beta-globin (HBG) as described in Materials and Methods. Data are representative of three independent experiments.


A
Catalytic domain
DNA binding domain
UDG
85
110
195
Leucine loop
235
EBV (BKRF3) VVILGQDPYHG-GQANGLAFSVAYGF GDKASLINSKKHLVLTSQHPSPLAQNSTRKSAQQKFLGNNH
HHV8 (ORF46) VVILGQDPYHK-GQATGLAFSVDPQC IDRTPLINAQKHLVLTAQHPSPLASLGGRHSRWPRFQGCNH
MHV68 (ORF46) VVILGQDPYHG-GQATGLAFSVHENF IEKEVLINGSKHLVLKAQHPSPLASLGGYSSKQAPFLGCNH
HCMV (UL114) VVIVGQDPYCD-GSASGLAFGTLAGR HTCEYLIDRRRHLVLKSCHPSP-------RNTTRAFVGNDH
VZV (ORF59) VVIIGQDPYPTAGHAHGLAFSVKRGI QKTTQP-NSRCHLVLTHAHPSPL-------SRVP-FRNCRH
HSV-1 (UL2) VVIIGQDPYHHPGQAHGLAFSVRANV QNAIRP-DPRVHCVLKFSHPSPL-------SKVP-FGTCQH
E.coli (UDG) VVILGQDPYHGPGQAHGLAFSVRPGI QKKGAIIDKQRHHVLKAPHPSPL-------SAHRGFFGCNH
Homo (UNG2) VVILGQDPYHGPNQAHGLCFSVQRPV QKKGSAIDRKRHHVLQTAHPSPL-------SVYRGFFGCRH
Catalytic domain
DNA binding domain
(Q90L,D91N) VVILGLNPYHG.GQANGLAFSVAYGF
(H213L) GDKASLINSKKHLVLTSQLPSPLAQNSTRKSAQQKFLGNNH
B
BKRF3STOP
p2089
Vec
HA-BKRF3
(Q90L, D91N) (H213L)
Dox - + - + - + - + - +
Zta
Rta
BMRF1
BALF2
BGLF4
HA-BKRF3
GAPDH
1 2 3 4 5 6 7 8 9 10
C
Neg. control
E. coli UDG
IP: HA
HA-BKRF3
vec
WT
(Q90L, D91N)
(H213L)
Dox - - - + - + - + - +
45 mer
20 mer
1 2 3 4 5 6 7 8 9 10
HA-BKRF3
Ig L
relative BKRF3 - - 1 1.3 1.3 1.4 0.8 1.2
normalized cleavage % - - 6 16 1 1 1 1
D
HA-BKRF3 WT
(Q90L, D91N)
(H213L)
% of DNA binding
FT
0.1
0.2
0.3
0.4
0.5
NaCl (M)
E
BKRF3STOP-PL1
BKRF3STOP-PL5
BKRF3STOP-PL11
HA-BKRF3
WT
(Q90L, D91N)
(H213L)
Dox
BamW
HBG
EBV DNA copy number/ cell
HA-BKRF3
Rta
Zta
BMRF1
UNG2
β-actin
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15

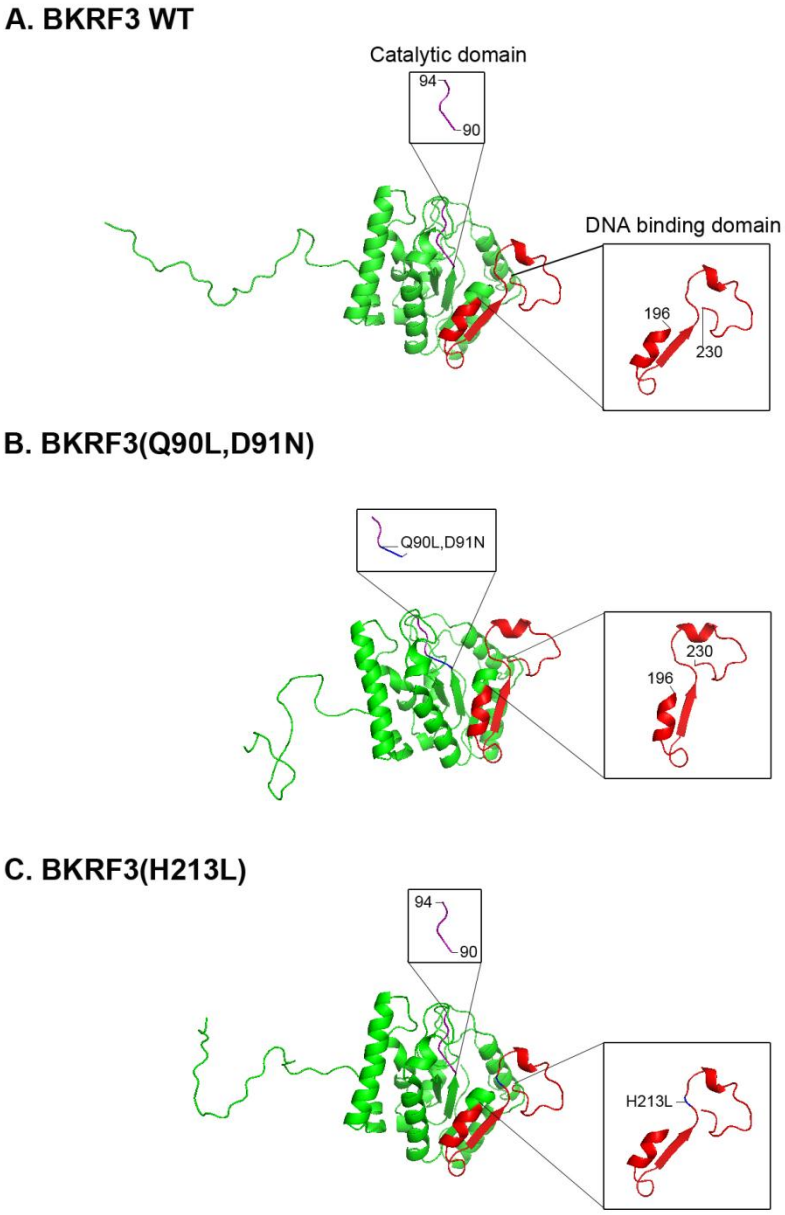


Fig. 12. The predicted 3D structure of BKRF3 wild-type and mutants.

(A) The 3D structure of BKRF3 was predicted by Protein Structure Prediction Server (PS)2-v2 (http://ps2.life.nctu.edu.tw) using BKRF3 fragment (residue 25 to 255) as template (PDB:2J8X.A) (Geoui et al. 2007). The catalytic and DNA binding domains of BKRF3 are showed in enlarged 3D images. The mutation sites of BKRF3(Q90L,D91N) and BKRF3(H213L) are showed in (B) BKRF3(Q90L,D91N) and (C) BKRF3(H213L).

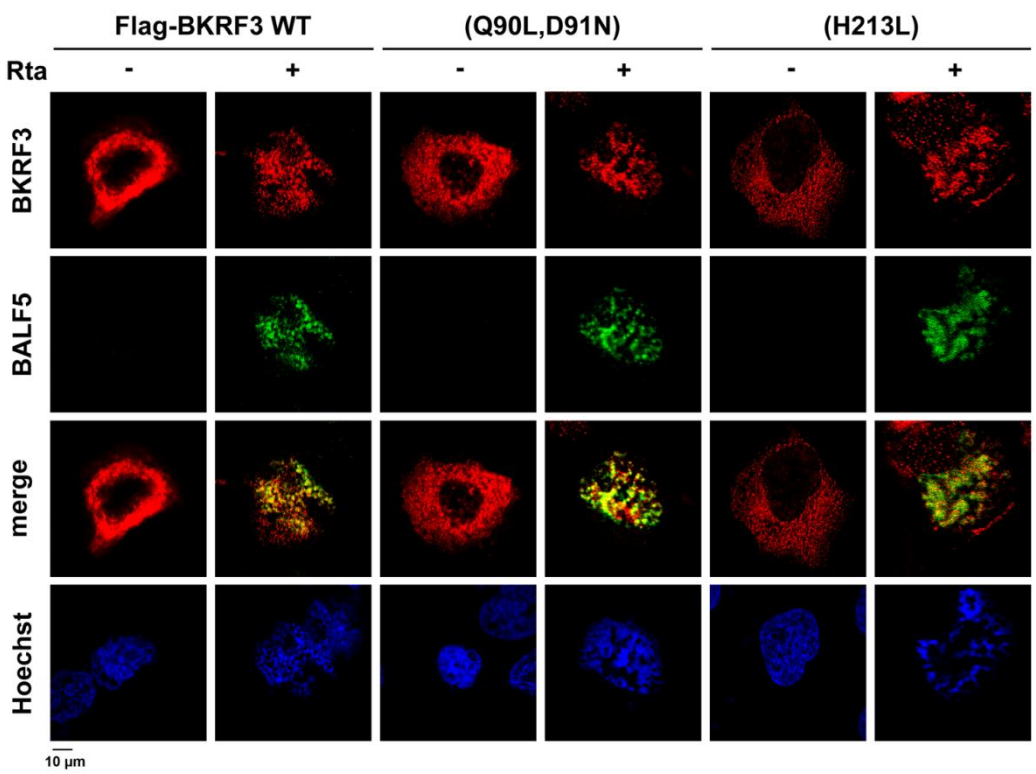


Fig. 13. Point mutation at His213 of BKRF3 attenuated its recruitment to the viral DNA replication compartment.

NA cells were seeded at 3×10^6 cells per 10-cm dish for slide culture and transfected with Flag-tagged wild-type BKRF3 (WT), BKRF3(Q90L,D91N), or BKRF3(H213L) plasmid combined with vector or Rta expression plasmid. At 48 h posttransfection, cells were fixed with 4% paraformaldehyde and stained for BKRF3, BALF5, and DNA with mouse anti-Flag antibody, rabbit BALF5 antiserum, and Hoechst 33258, followed by confocal microscopy analysis.

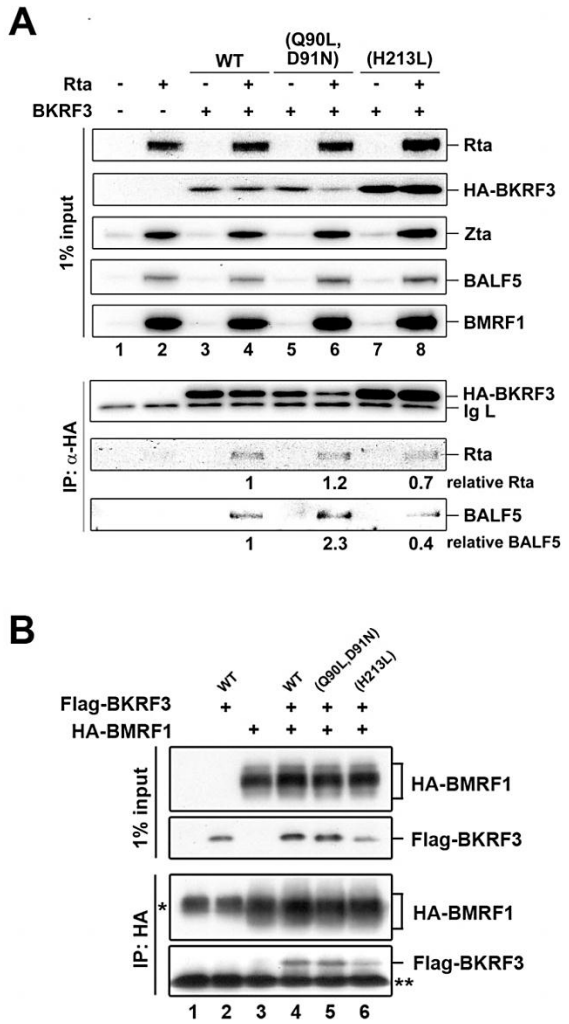


Fig. 14. Point mutation at His213 of BKRF3 attenuated its ability to interact with the viral DNA polymerase BALF5 and immediate-early gene, Rta.

(A) To measure the ability of wild-type or mutant BKRF3 to interact with viral DNA replication-associated proteins, NA cells transfected with 7, 14, or 4 μg of HA-BKRF3 WT, BKRF3(Q90L,D91N), or BKRF3(H213L) were induced into the lytic cycle by Rta transfection. At 60 h posttransfection, cell lysates were harvested for coimmunoprecipitation assay. The HA-BKRF3-associated complexes were precipitated with anti-HA and detected with anti-Rta, anti-BALF5, and anti-HA antibodies by Western blotting. This is a representative result of triplicate experiments. (B) To verify the interacting abilities of BMRF1 with BKRF3 WT, BKRF3(Q90L,D91N), and BKRF3(H213L), 293T cells were cotransfected with expression plasmids as indicated for 48 h. Whole-cell lysates were subjected to a coimmunoprecipitation assay. The HA-BMRF1-associated protein complexes were precipitated with anti-HA and detected with anti-Flag by Western blotting. This is a representative result of duplicate experiments. *, Ig heavy chain; **, Ig light chain.

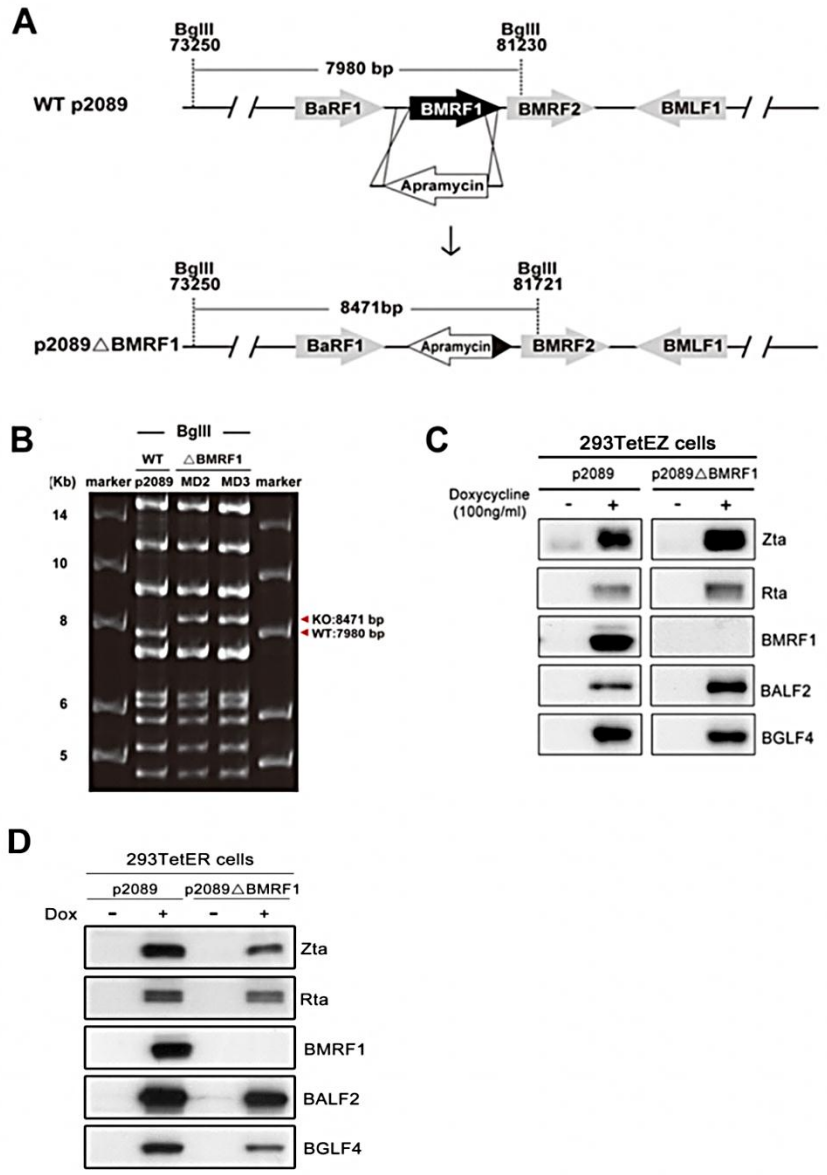
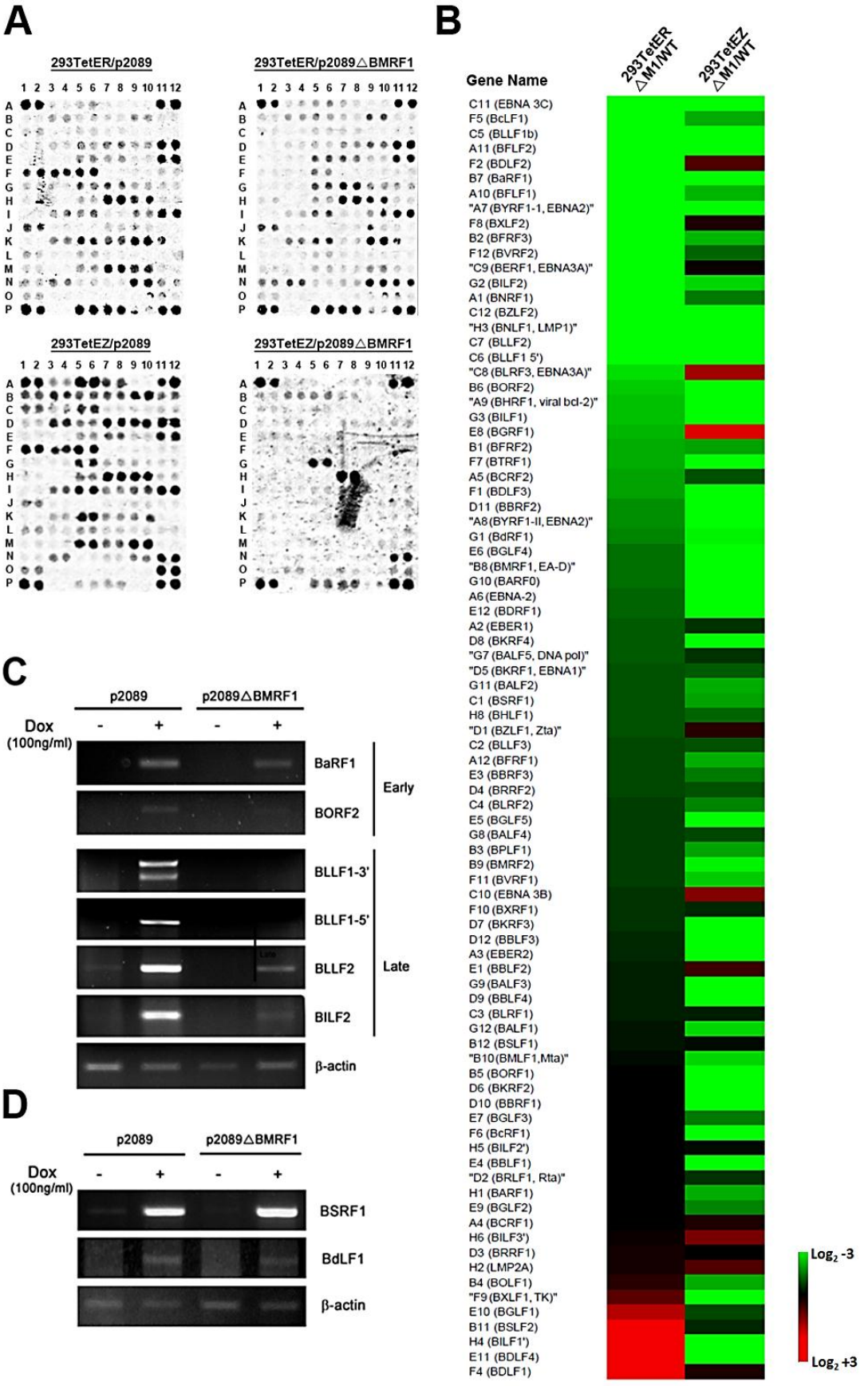


Fig. 15. Construction of BMRF1 knockout EBV bacmid and establishment of inducible bacmid 293TetER cell lines.

(A) To generate Maxi-EBV△BMRF1 bacmid, the BMRF1 ORF of Maxi-EBV p2089 was replaced with apramycin resistant gene by PCR targeting. After homologous recombination, the size of BamHI-M fragment was increased from 7,980 bp to 8,471 bp. (B) Maxi-EBV p2089 wild-type and p2089△BMRF1 bacmid DNA were digested by BglII, and the genome pattern within wild-type and BMRF1 mutant were analyzed by pulsed field gel electrophoresis. (C and D) The wild-type or BMRF1 knockout bacmid was transfected into (C) 293TetEZ or (D) 293TetER cell and selected with hygromycin (50 μg/ml) for one month. Selected bacmid cell lines were treated with doxycycline (100 ng/ml) to induce lytic cycle progression. Cell lysates from 293TetER/p2089 and 293TetER/p2089△BMRF1 cells were harvested for western blotting analysis.

Fig. 16. Effects of BMRF1 on viral gene expression in Rta or Zta inducible 293 cells.

(A) Total RNA harvested from doxycycline-treated cells was reversely transcribed and labeled with biotin-dUTP for EBV DNA microarray assay as described in Materials and Methods. After color development, chromogenic spots on the membranes were scanned and converted to digital signals for quantification. (B) The down-regulated folds of EBV genes affected by BMRF1 in Rta or Zta inducible cell lines were converted to log base 2. (C and D) The expressions of EBV lytic genes were detected by RT-PCR. (C) EBV early genes and late genes that were affected in 293TetER/p2089△BMRF1 cells. (D) EBV late genes which showed no difference on EBV DNA microarray analysis between p2089 wild-type and △BMRF1 cells also are detected in RT-PCR assays.


A
293TetER/p2089
293TetER/p2089△BMRF1
293TetEZ/p2089
293TetEZ/p2089△BMRF1
B
Gene Name
293TetER △M1/WT
293TetEZ △M1/WT
C11 (EBNA 3C)
F5 (BcLF1)
C5 (BLLF1b)
A11 (BFLF2)
F2 (BDLF2)
B7 (BaRF1)
A10 (BFLF1)
"A7 (BYRF1-1, EBNA2)"
F8 (BXLF2)
B2 (BFRF3)
F12 (BVRF2)
"C9 (BERF1, EBNA3A)"
G2 (BILF2)
A1 (BNRF1)
C12 (BZLF2)
"H3 (BNLF1, LMP1)"
C7 (BLLF2)
C6 (BLLF1 5')
"C8 (BLRF3, EBNA3A)"
B6 (BORF2)
"A9 (BHRF1, viral bcl-2)"
G3 (BILF1)
E8 (BGRF1)
B1 (BFRF2)
F7 (BTRF1)
A5 (BCRF2)
F1 (BDLF3)
D11 (BBRF2)
"A8 (BYRF1-II, EBNA2)"
G1 (BdRF1)
E6 (BGLF4)
"B8 (BMRF1, EA-D)"
G10 (BARF0)
A6 (EBNA-2)
E12 (BDRF1)
A2 (EBER1)
D8 (BKRF4)
"G7 (BALF5, DNA pol)"
"D5 (BKRF1, EBNA1)"
G11 (BALF2)
C1 (BSRF1)
H8 (BHLF1)
"D1 (BZLF1, Zta)"
C2 (BLLF3)
A12 (BFRF1)
E3 (BBRF3)
D4 (BRRF2)
C4 (BLRF2)
E5 (BGLF5)
G8 (BALF4)
B3 (BPLF1)
B9 (BMRF2)
F11 (BVRF1)
C10 (EBNA 3B)
F10 (BXRF1)
D7 (BKRF3)
D12 (BBLF3)
A3 (EBER2)
E1 (BBLF2)
G9 (BALF3)
D9 (BBLF4)
C3 (BLRF1)
G12 (BALF1)
B12 (BSLF1)
"B10 (BMLF1,Mta)"
B5 (BORF1)
D6 (BKRF2)
D10 (BBRF1)
E7 (BGLF3)
F6 (BcRF1)
H5 (BILF2')
E4 (BBLF1)
"D2 (BRLF1, Rta)"
H1 (BARF1)
E9 (BGLF2)
A4 (BCRF1)
H6 (BILF3')
D3 (BRRF1)
H2 (LMP2A)
B4 (BOLF1)
"F9 (BXLF1, TK)"
E10 (BGLF1)
B11 (BSLF2)
H4 (BILF1')
E11 (BDLF4)
F4 (BDLF1)
Log2 -3
Log2 +3
C
p2089
p2089△BMRF1
Dox (100ng/ml)
BaRF1
BORF2
Early
BLLF1-3'
BLLF1-5'
BLLF2
BILF2
Late
β-actin
D
p2089
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Dox (100ng/ml)
BSRF1
BdLF1
β-actin

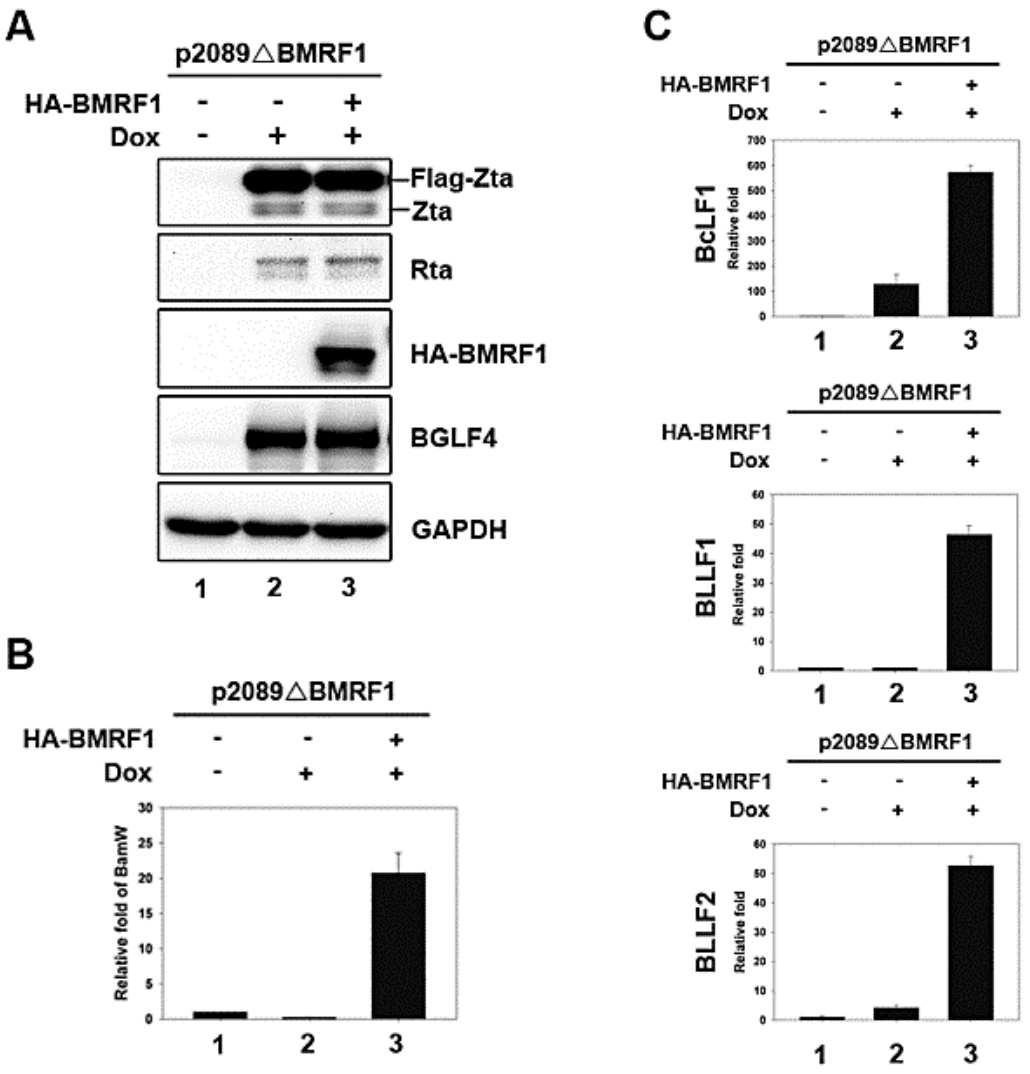


Fig. 17. Ectopic expression of BMRF1 rescues viral DNA replication and transcription of viral genes BcLF1, BLLF1 and BLLF2 in 293TetEZ/p2089△BMRF1 cell.

In trans-complementation assay, the 293TetEZ/p2089△BMRF1 cells were transfected with HA-BMRF1 expressing plasmid for 24 h, and cells were treated with or without doxycycline (Dox, 50 ng/ml) for another 48 h. After complementation of BMRF1 and lytic induction by doxycycline, cells were harvested for (A) lytic proteins analysis by western blotting, (B) EBV DNA copy number detected by Q-PCR as described in Materials and Methods and (C) viral transcripts detected by RT-qPCR.

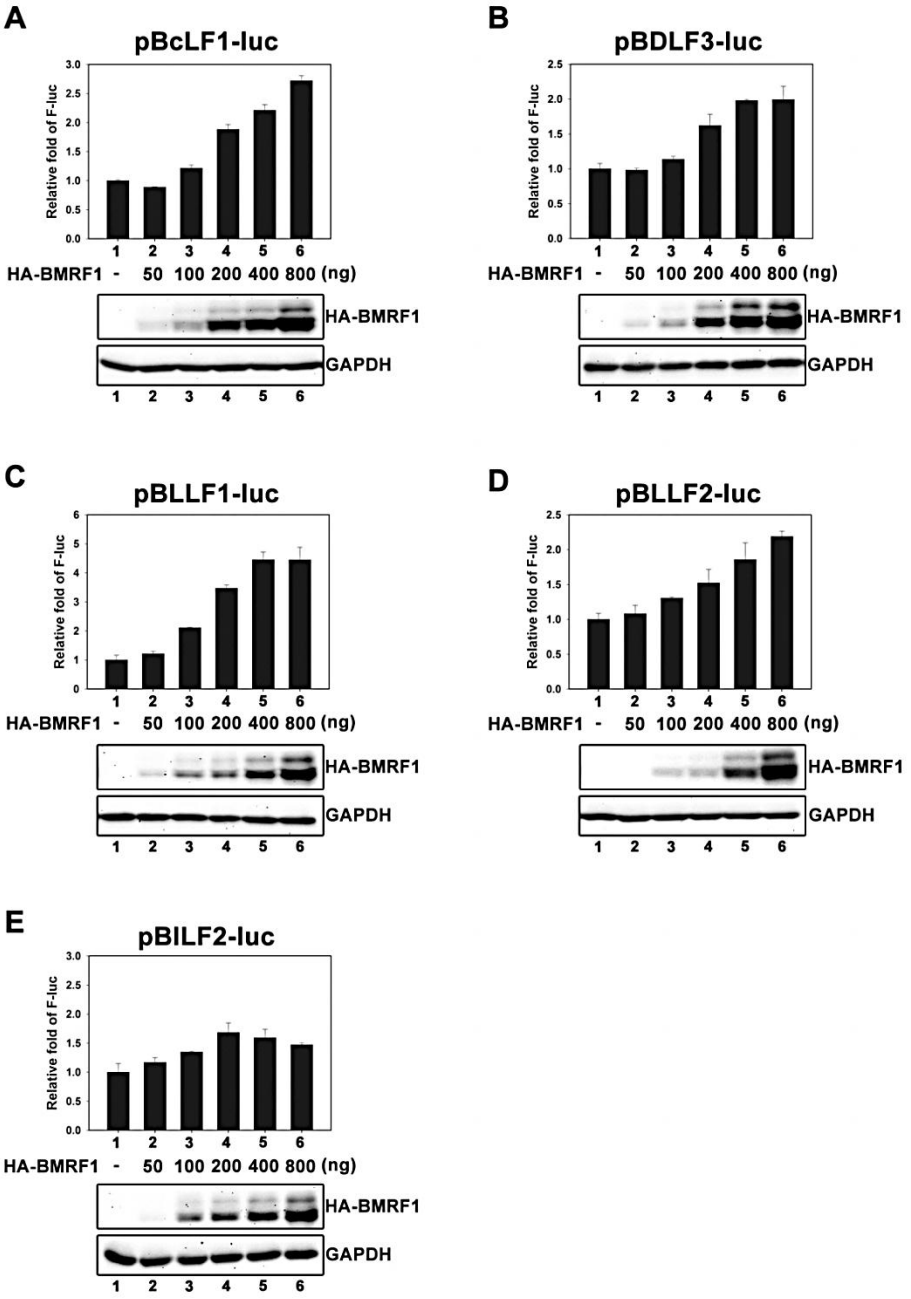


Fig. 18. Expression of BMRF1 alone turns on the promoter activities of EBV late genes in 293T cells.

The 293T cells (1×10^5 cells) were seeded into a 12-well culture plate and co-transfected with indicated increasing doses of BMRF1 expressing plasmids with the reporter plasmids, including (A) pGL2-BcLF1, (B) pGL2-BDLF3, (C) pGL2-BLLF1, (D) pGL2-BLLF2 and (E) pGL2-BILF2 by BBS for 48 h. In all reporter assays, the Renilla Luc plasmid (PRL-null, 250 ng/well) was co-transfected as an internal control. The luciferase activities were detected by a luminometer using Dual-Glo® Luciferase Assay kit.

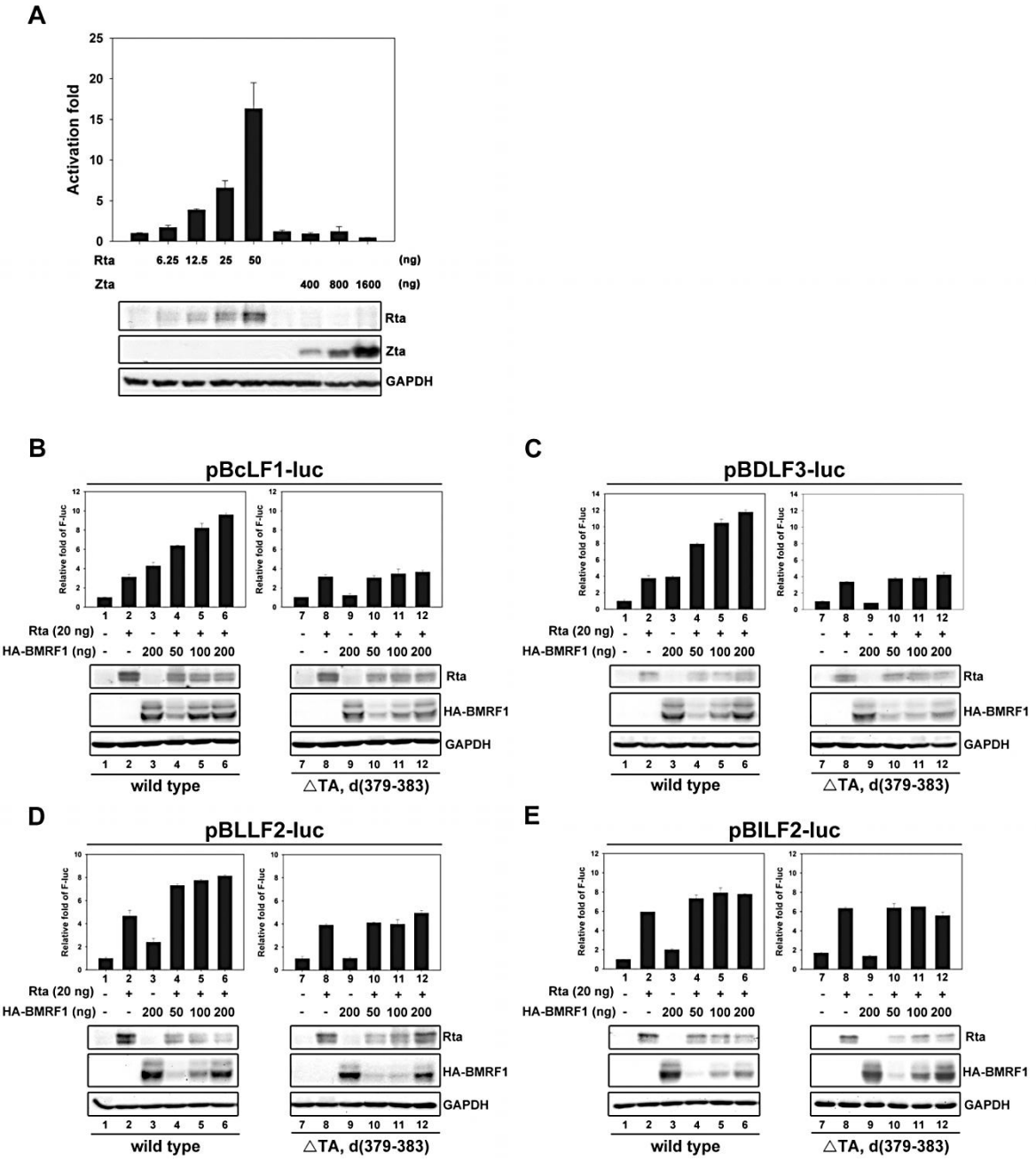
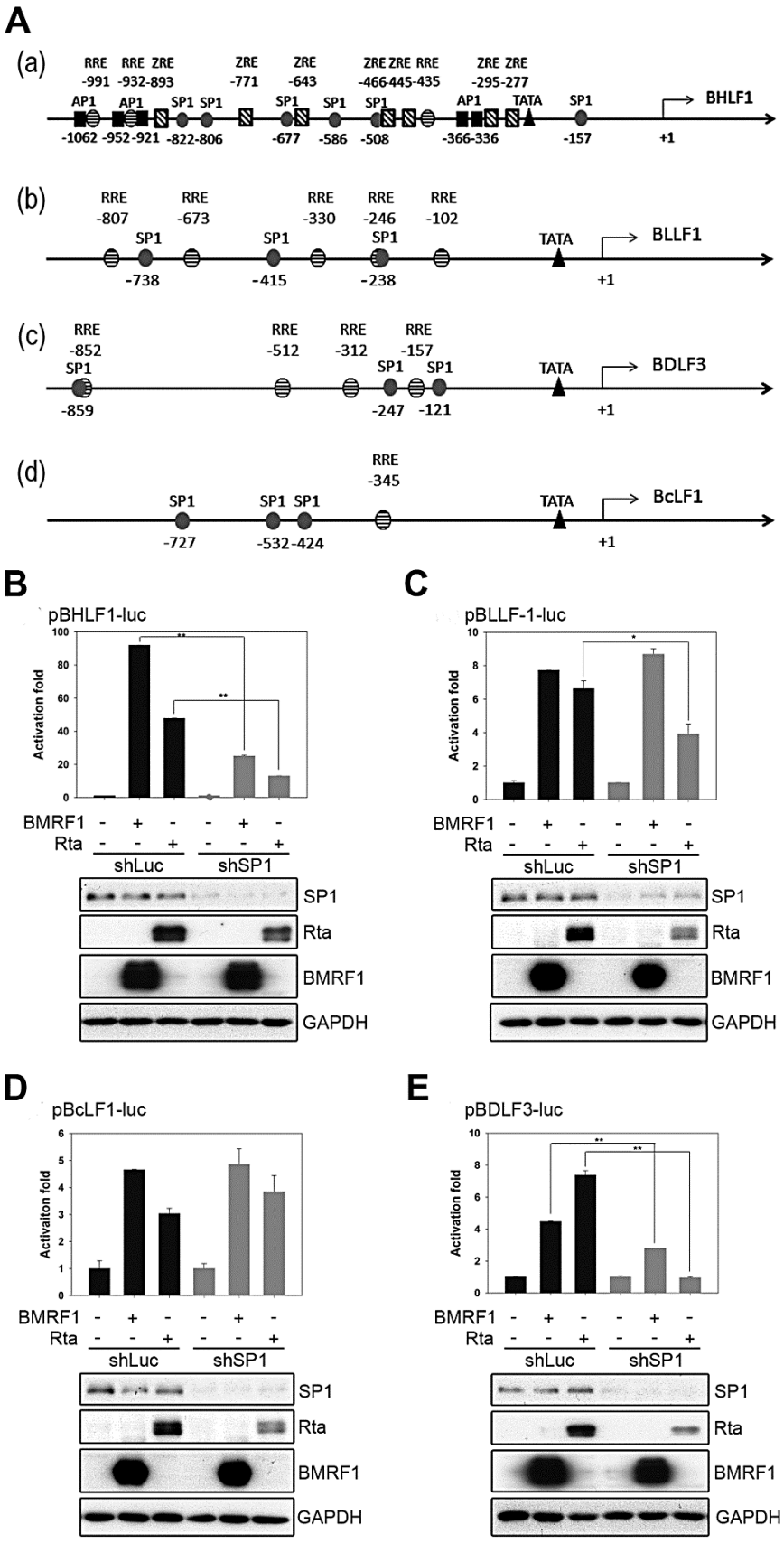


Fig. 19. BMRF1 enhances Rta-mediated transcriptional activities through its transactivation domain.

(A) Different dosages of Rta or Zta expressing plasmid was co-transfected with pGL2-BLLF1 and PRL-null plasmids into 293T cells. At 48 h posttransfection, the luciferase activities were detected. (B to E) The indicated expressing plasmids with 400 ng reporter plasmid, including (B) pGL2-BcLF1, (C) pGL2-BDLF3, (D) pGL2-BLLF2 and (E) pGL2-BILF2 were co-transfected into 293T cells. At 48 h posttransfection, luciferase activities were detected by luciferase assay.

Fig. 20. BMRF1 regulates the promoter activity of BDLF3 through a SP1-dependent pathway.

(A) The SP1 or AP1 binding sites of (a) BHLF1, (b) BLLF1, (c) BDLF3 and (d) BcLF1 were predicted by TFSEARCH (ver 1.3). The ZREs (TG/T-T/A-G-T/C-G/C/A-A) or RREs ($GNCCN_9GGNG$) are shown on the upper panel of promoter regions. N stands for any nucleotide. The triangle indicates TATA box. (B to E) 293T cells were transduced with shLuc or shSP1 lentivirus for 2 days and were selected with puromycin (3 μg/ml) for 5 days. The shLuc control or shSP1 knockdown cells were co-transfected with indicatied effector plasmids and reporter plasmid, including (B) pGL2-BHLF1, (C) pGL2-BLLF1, (D) pGL2-BcLF1 and (E) pGL2-BDLF3 for luciferase assay.


A
(a)
RRE -991
RRE -932
ZRE -893
ZRE -771
ZRE -643
ZRE -466
ZRE -445
RRE -435
ZRE -295
ZRE -277
AP1 -1062
AP1 -952
-921
SP1 -822
SP1 -806
SP1 -677
SP1 -586
SP1 -508
AP1 -366
-336
TATA
SP1 -157
+1
BHLF1
(b)
RRE -807
RRE -673
RRE -330
RRE -246
RRE -102
SP1 -738
SP1 -415
SP1 -238
TATA
+1
BLLF1
(c)
RRE -852
SP1 -859
RRE -512
RRE -312
RRE -157
SP1 -247
SP1 -121
TATA
+1
BDLF3
(d)
RRE -345
SP1 -727
SP1 -532
SP1 -424
TATA
+1
BcLF1
B
pBHLF1-luc
Activation fold
BMRF1
Rta
shLuc
shSP1
SP1
Rta
BMRF1
GAPDH
C
pBLLF-1-luc
Activation fold
BMRF1
Rta
shLuc
shSP1
SP1
Rta
BMRF1
GAPDH
D
pBcLF1-luc
Activaiton fold
BMRF1
Rta
shLuc
shSP1
SP1
Rta
BMRF1
GAPDH
E
pBDLF3-luc
Activation fold
BMRF1
Rta
shLuc
shSP1
SP1
Rta
BMRF1
GAPDH

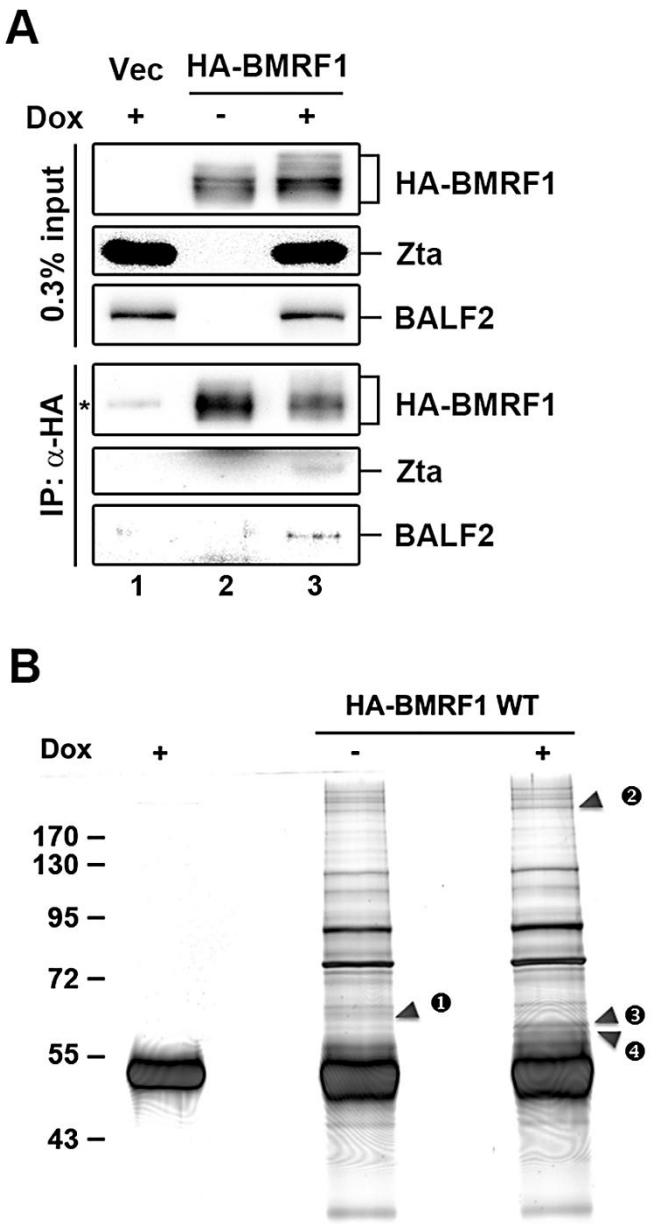


Fig. 21. Determination of BMRF1 interacting proteins.

Cell lysates harvested from 293TetEZ/p2089△BMRF1 cells that were transfected with HA-BMRF1 expressing plasmid and treated with or w/o doxycycline (Dox) for 48 h were used for immunoprecipitation with anti-HA antibody. One tenth of the captured immunocomplexes were analyzed by (A) western blotting assay and 90% of captured products were subjected to (B) silver staining. The indicated bands on the silver stained gel were subjected to mass spectrometry analysis. BMRF1 interacting proteins identified in mass spectrometry analysis were sub-grouped as shown in Table 3. *, Ig heavy chain.

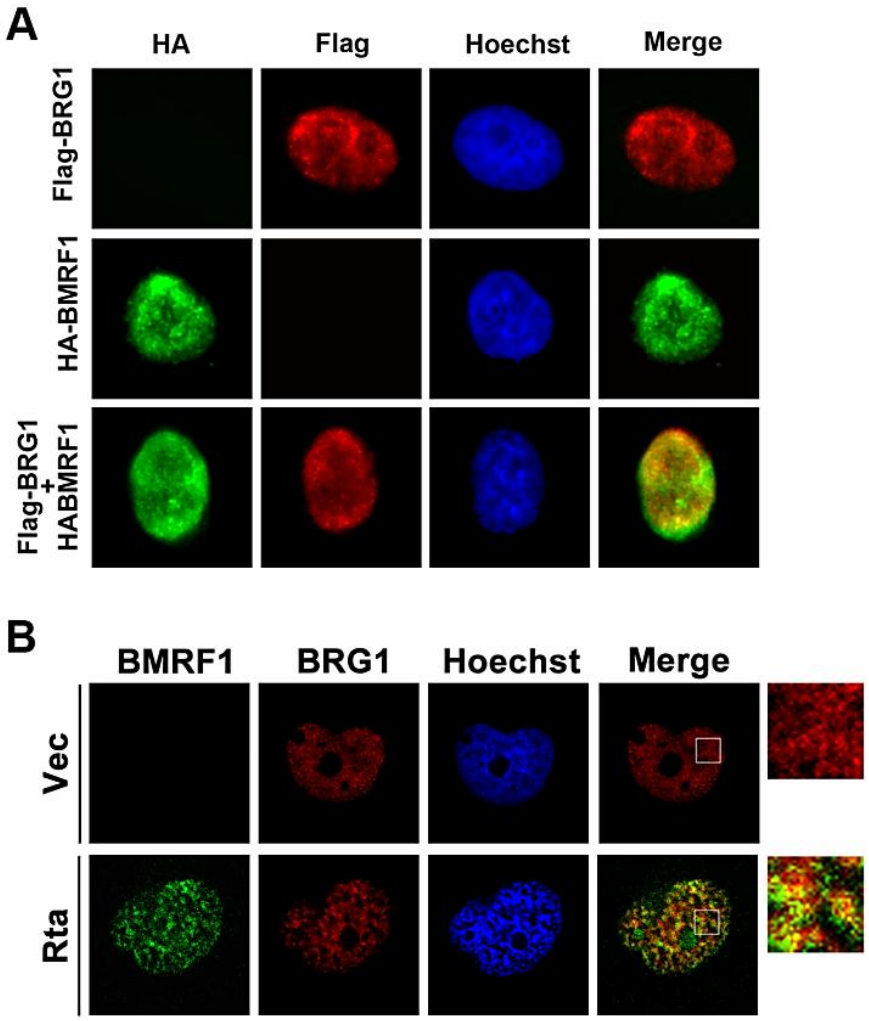


Fig. 22. Nuclear co-localization of BMRF1 and chromatin modifier BRG1.

(A) The Flag-BRG1 expressing plasmid was co-transfected with vector control of HA-BMRF1 into HeLa cells for immunefluorescence analysis. The distribution of BRG1 and BMRF1 were detected by mouse anti-Flag and rabbit anti-HA antibody, respectively. (B) At 48 h postinduction, slide cultured NA cells were harvested for immunofluorescent analysis using mouse anti-BMRF1 and rabbit anti-BRG1 antibody. The distribution of BMRF1 and BRG1 were observed by confocal microscopy.

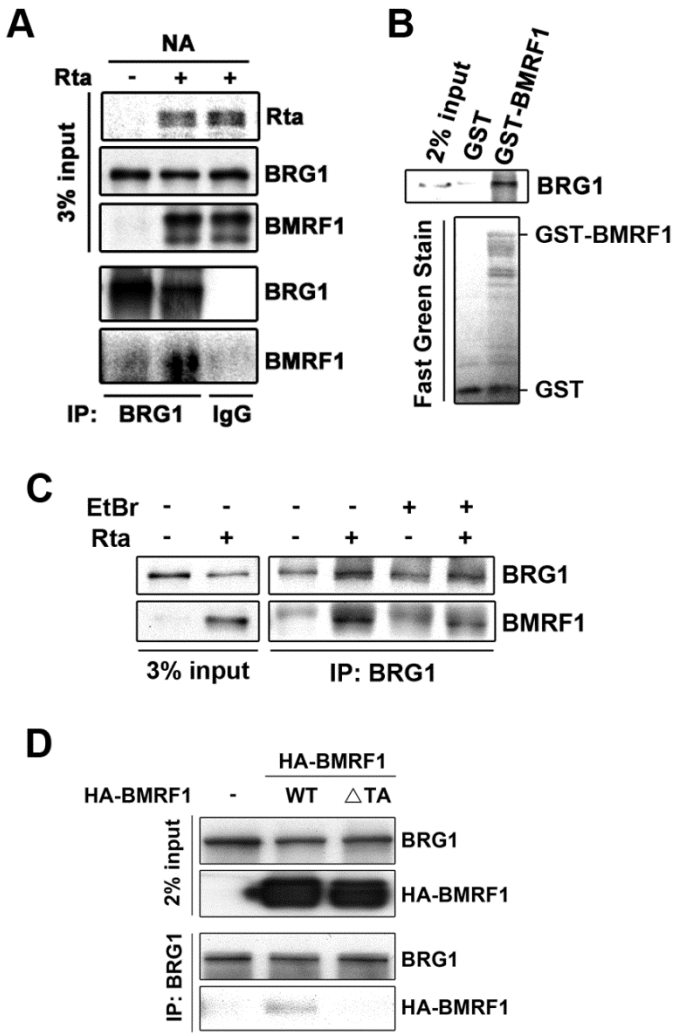


Fig. 23. BMRF1 associates with BRG1 in coimmunoprecipitation and in GST-pull down assays.

(A and C) Cell lysates harvested from control vector or Rta transfected NA cells were pretreated with or w/o EtBr (10 μg/ml) on ice for 30 min. Pretreated cell lysates were incubated with anti-BRG1 or control IgG to precipitated BRG1-associated complexes and subjected to western blotting analysis. (B) Glutathione beads binding with bacterially expressed GST or GST-BMRF1 were incubated with 293T cell lysates at 4°C for 18 h. After washed with GST pull-down washing buffer, the pull-down complexes were resolved by SDS-PAGE and followed by western blot analysis. (D) The vector control, HA-BMRF1 wild-type or transactivation domain mutant plasmid (△TA) was transfected into 293T cells for 48 h. The transfected cells lysates were harvested and subjected to immunoprecipitation assay using mouse anti-BRG1 antibody.

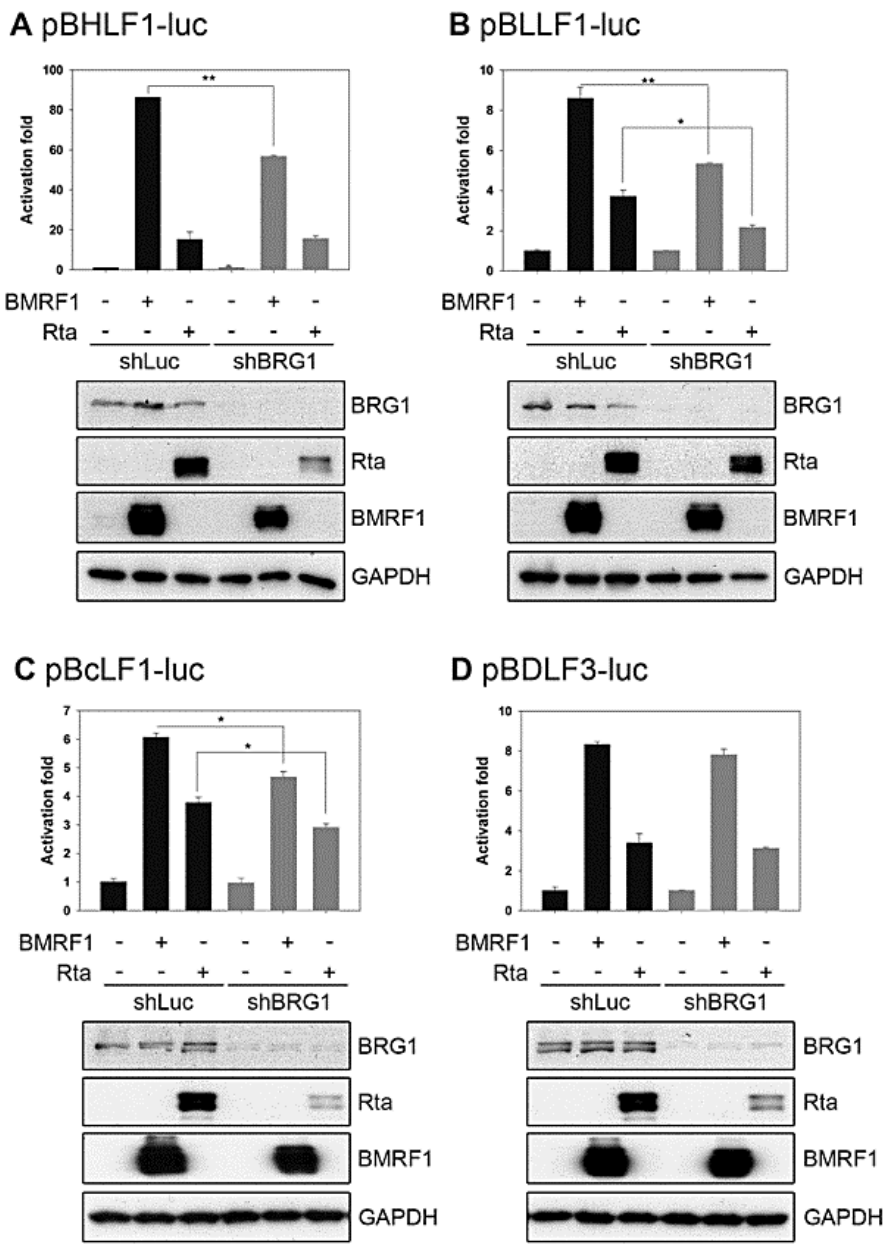


Fig. 24. Knockdown of BRG1 reduces the transactivation activity of BMRF1 on the promoters of BHLF1, BLLF1 and BcLF1.

293T cells were transduced with shLuc or shBRG1 lentivirus for 48 h and selected with puromycin (3μg/ml) for 5 days. The shLuc control and BRG1 knockdown cells were co-transfected with indicatied effector and reporter plasmids, including (A) pGL2-BHLF1, (B) pGL2-BLLF1, (C) pGL2-BcLF1 and (D) pGL2-BDLF3 by calcium phosphate-BES method. At 48 h posttransfection, the luciferase activities and protein expression were detected by reporter assay and western blotting analysis, respectively.

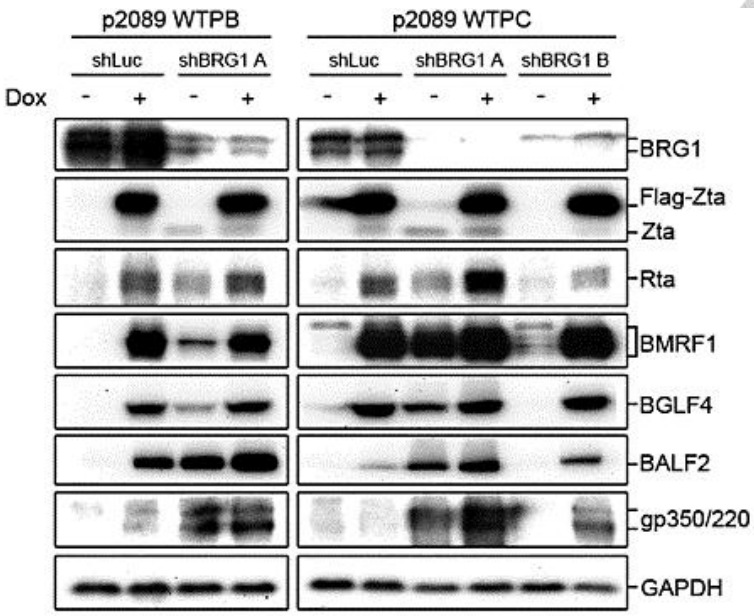
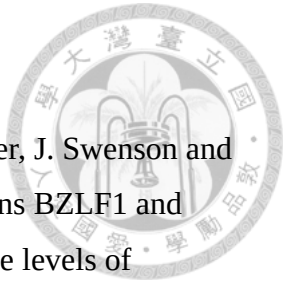


Fig. 25. BRG1 knockdown induced spontaneous EBV reactivation in 293TetEZ/p2089 wild-type cells.

293TetEZ/p2089 wild-type pool clone-B and -C cells (WTPB and WTPC) were transduced with shLuc or shBRG1 lentivirus and selected with puromycin (3μg/ml) for 10 days. The selected cells were subsequently induced Flag-Zta expression by doxycycline for 60 h. After doxycycline (Dox, 50 ng/ml) induction, cell lysates were harvested for lytic protein detection by western blotting analysis.



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