Title: **BS69 negatively regulates the canonical NF-κB activation induced by Epstein-Barr virus-derived LMP1**

Authors: Osamu Ikeda[1], Yuichi Sekine[1], Akihiro Mizushima[1], Kenji Oritani[2], Teruhito Yasui[3], Masahiro Fujimuro[4], Ryuta Muromoto[1], Asuka Nanbo[1], and Tadashi Matsuda[1,*]

Affiliation: [1]Department of Immunology, Graduate School of Pharmaceutical Sciences Hokkaido University, Sapporo 060-0812 Japan, [2]Department of Hematology and Oncology, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan, [3]Department of Molecular Immunology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan, [4]Department of Molecular Cell Biology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Chuo 409-3898, Japan.

*****Address for manuscript correspondence:* Dr. Tadashi Matsuda, Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-Ku Kita 12 Nishi 6, Sapporo 060-0812, Japan TEL: 81-11-706-3243, FAX: 81-11-706-4990, E-mail: tmatsuda@pharm.hokudai.ac.jp

Abstract

Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) activates NF-κB signaling pathways through the two C-terminal regions, CTAR1 and CTAR2. BS69 has previously been shown to be involved in LMP1-induced c-Jun N-terminal kinase activation through CTAR2 by interacting with tumor necrosis factor (TNFR) receptor-associated factor 6. In the present study, our experiments manipulating of BS69 expression clearly indicated that BS69 negatively regulated LMP1-mediated NF-κB activation including up-regulated IL-6 mRNA expression and IκB degradation. For the mechanisms, our immunoprecipitation experiments proposed that BS69 decreased complex formation between LMP1 and TNFR-associated death domain protein (TRADD).

Keywords: EBV, LMP1, BS69, NF-κB, TRADD

1. Introduction

Epstein–Barr virus (EBV) is linked to the development of multiple malignancies, including post-transplant lymphoma, Hodgkin disease, and nasopharyngeal carcinoma. EBV-encoded latent membrane protein 1 (LMP1) is expressed in many EBV-associated tumor cells and is responsible for most of the altered cellular growth properties. Structurally, LMP1 is an integral membrane protein of 386 amino acids (aa) that consists of a short cytoplasmic N-terminal domain (aa 1-24), six transmembrane domains (aa 25-186) and a long cytoplasmic C-terminal tail (aa 187-386) [1,2,3]. The cytoplasmic C-terminal tail contains two C-terminal activation regions (CTARs), CTAR1 and CTAR2, which are required for LMP1-transduced signaling through tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) [1,4]. CTAR1 contains a consensus TRAF-binding motif (PXQXT), and can bind to TRAFs 1, 2, 3 and 5 [1,5-8]. CTAR2 interacts with TNFR-associated death domain protein (TRADD), receptor-interacting protein (RIP) [9,10] and BS69 [11]. Signals through these two domains induce p100 and p105 NF-κB precursors and generate p52 and p50 subunits, respectively [12,13], indicating that LMP1 is involved in both canonical and non-canonical activation of NF-κB pathway. Binding of LMP1 to TRAFs and TRADD also

initiates the formation of a signaling complex that leads to activation of mitogen-activated protein kinase, p38 [14]. In addition, activation of c-Jun N-terminal kinase (JNK) by LMP1 is mediated through CTAR1 and CTAR2 [15].

BS69, a multidomain cellular protein containing PHD, Bromo, PWWP and MYND domains, was originally identified as an adenovirus E1A-binding protein that inhibits the transactivation function of E1A [16]. Furthermore, the C-terminal MYND domain of BS69 was shown to bind to the PXLXP motif existing on E1A, the EBV-encoded EBNA2 and a Myc-related cellular protein MGA [17]. It has recently been shown that BS69 interacts with EBV-encoded LMP1, through its MYND domain and acts as a scaffold protein in the LMP1-mediated JNK pathway [11] by interacting with TRAF6. However, the involvement of BS69 in LMP1-mediated NF-κB activation is still undetermined. Here, we investigated the influence of BS69 on LMP1-mediated NF-κB activation.

Our main finding was that BS69 could downregulate LMP1-mediated NF-κB activation including LMP1-induced interleukin (IL)-6 mRNA expression and IκB degradation. We further found that BS69 decreases complex formation between LMP1 and TRADD. Therefore, BS69 is likely to act as an endogenous negative regulator of LMP1-mediated NF-κB activation by displacing TRADD from LMP1.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant human TNF-α was kindly provided from Dainippon Sumitomo Pharma (Osaka, Japan). Expression vectors for BS69, BRAM1, TRADD and NF-κB-LUC were kindly provided by H. Shibuya (Tokyo Medical and Dental University, Tokyo, Japan), N. Inohara (University of Michigan Medical School, Ann Arbor, MI) and T. Fujita (Kyoto University, Kyoto, Japan)[18- 20]. Epitope-tagged STAP-2 and epitope-tagged LMP1 constructs were described previously [19, 20] The following primary antibodies were obtained commercially: anti-TRADD, anti-NF-κB p65 and anti-Myc antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); anti-FLAG mAb (M2) and anti-HA antibody (Sigma-Aldrich, St. Louis, MO); anti-IκB antibody, anti-phospho-JNK (pJNK) and anti-JNK (Cell Signaling Technology, Beverly, MA); and anti-actin mAb (Chemicon International, Temecula, CA). An anti-LMP1 mAb (S12) was prepared as described previously [19].

2.1. Cell culture and transfection

A human cervix carcinoma cell line (HeLa), human embryonic kidney carcinoma cell line (293T) and human hepatoma cell line (Hep3B) were maintained in DMEM containing 10% FCS. HeLa/pcDNA3 and HeLa/STAP-2 transfectants were prepared as described previously [19]. HeLa cells were transfected using jetPEI (PolyPlus-transfection, Strasbourg, France) according to the manufacturer's instructions. 293T cells were transfected using a standard calcium precipitation protocol [21].

2.2. Transfection of small-interfering RNAs (siRNAs) and luciferase assays

The siRNAs targeting human BS69 (#1 and #2) used in this study were as follows: BS69#1, 5'-GGAUGAAGUCGGACCACAATT-3'; BS69#2, 5'-GAAGUUAUGAAGAGUUCAATT-3'. HeLa and Hep3B cells were plated on 24-well plates at $2x10^4$ cells/well and incubated with an siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37°C for 4 h, followed by the addition of fresh medium containing 10% FCS. HeLa cells were further transfected with or without NF-□B-LUC with or without LMP1 using jetPEI as described in the section above. At 24 h after transfection, the cells were harvested and assayed for their luciferase activities using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the

manufacturer's instructions. 293T cells were transfected as described in the section above. The cells were harvested at 36 h after transfection, lysed in 50 μl of Reporter Lysis Buffer (Promega) and assayed for their luciferase and β-galactosidase activities according to the manufacturer's instructions. Three or more independent experiments were carried out for each assay.

2. 3. Immunoprecipitation and immunoblotting

Immunoprecipitation and western blotting assays were performed as described previously [21]. The immunoprecipitates from cell lysates were resolved by SDS-PAGE and transferred to PVDF membranes (PerkinElmer, Boston, MA). The membranes were then immunoblotted with the different primary antibodies. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore, Bedford, MA).

2. 4. RNA isolation, RT-PCR and quantitative real-time PCR

After cell harvesting, total RNA samples were extracted using Iso-Gen (Nippon Gene, Tokyo, Japan) and subjected to RT-PCR using an RT-PCR High -Plus- Kit (TOYOBO, Tokyo, Japan)[19]. Primers used for RT-PCR were: BS69: 5'-

GGATATTGGTTGCCAGGA GA-3' (sense), 5'-GTGCCCATCTCCTGTTTGTT-3' (antisense). Quantitative real-time PCR analyses for IL-6, BS69 and control G3PDH mRNA transcripts were carried out using Assays-on-Demand^{TM} gene-specific fluorescently-labeled TaqMan MGB probes in an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA).

2.5. Indirect immunofluorescence microscopy

To analyze the subcellular localization of LMP1 and BS69 proteins, FLAG-tagged LMP1 and HA-tagged BS69 were transiently transfected into HeLa cells by Jet-PEI. Immunofluorescence stainings were performed as described [19]. The following primary antibodies were used: mouse anti-HA and rabbit anti-FLAG antibodies. Two secondary antibodies were used: rhodamine-conjugated anti-mouse IgG or fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Chemicon). DNA was visualized with DAPI (Wako Chemicals, Osaka, Japan) staining. Confocal laser scanning microscopy was performed with a LSM510 microscope (Carl Zeiss, Thornwood, NY) with an Apochromat x63/1.4 oil immersion objective, using excitation wavelengths of 543 nm (rhodamine red) and 488 nm (FITC).

3. Results and discussion

3. 1. BS69 negatively regulates LMP1-induced NF-κB activation

We previously showed that STAP-2 suppressed LMP1-induced NF-κB activation and that overexpression of BS69 failed to affect the negative effects of STAP-2 [19]. However, information that BS69 can bind to the cytoplasmic C-terminal domain of LMP1 [11] let us investigate the individual effects of BS69 on LMP1-induced NF-κB activation using transient reporter assays with NF-κB-LUC. NF-κB-LUC activities were markedly induced by the expression of LMP1 in HeLa and 293T cells (Fig. 1). Importantly, the LMP1-induced NF-κB-LUC activities were significantly reduced by co-expression of BS69 in a dose-dependent manner, suggesting that BS69 negatively regulates LMP1-induced NF-κB activation. Recently, an alternatively spliced variant of BS69, BRAM1has also been shown to interact with the C-terminal domain of LMP1 and influence LMP1-mediated NF-κB activation [22]. BRAM1 protein shares the C-terminal 186 amino acids including a MYND domain with BS69 [18]. Thus, we examined the effect of BRAM1 on LMP1-induced NF-κB activation in the same situation. As shown in Fig, 1B, BRAM1 as well as BS69 decreased LMP1-induced NF-κB activation, and similar amounts of both proteins were required for this suppressive

effect. To exclude the possibility that the suppression of NF-κB activation is a result of promoter interference, the expression vector carrying TRAF1 cDNA was transfected into 293T cells, and LMP1-induced NF-κB activation was analyzed in those cells. As expected, overexpression of TRAF1 failed to influence NF-κB-LUC activity induced by LMP1 (Fig. 1C). We then tested the effect of ectopic exprerssion of BS69 or BRAM1 on NF-κB activation induced by TNF-α, which uses different signaling pathway from LMP1. As shown in Fig, 1D, neither BRAM1 nor BS69 overexpression reduced TNF-α-induced NF-κB-LUC activity in HeLa cells. Therefore, overexpression of BS69 negatively regulated LMP1-induced NF-κB activation. BS69 is shown to act as a scaffold protein in the LMP1-mediated JNK pathway [11]. We also examined the effect of BS69 and BRAM1 on LMP1-induced JNK activation using a phospho-specific anti-JNK antibody. As shown in Fig. 1E, ectopic expression of BS69 enhanced LMP1-induced JNK activation as previously reported [11]. However, BRAM1 expression had no effect on LMP1-induced JNK activation. Therefore, BS69 but not BRAM1 positively regulates LMP1-mediated JNK pathway.

To further explore whether BS69 represses LMP1-induced NF-κB activation, we used siRNAs to reduce endogenous expression of BS69 in HeLa cells (Fig. 2). HeLa cells were transfected with specific siRNAs for BS69 (#1 and #2) or a control siRNA. Total

RNA isolated from the transfected cells was subjected to RT-PCR and quantitative real-time PCR analysis, which confirmed a reduction of BS69 mRNA expression. We then determined the effects of BS69 siRNAs on LMP1-induced NF-κB activation in HeLa cells. As shown in Fig. 2B, siRNA-mediated reduced expression of BS69 resulted in a significant enhancement of LMP1-induced NF-κB-LUC activities in these cells. 293T cells express adenovirus E1A, and HeLa cells are known to express human papillomavirus E7 oncoprotein that is structurally and functionally related to E1A and may also bind BS69 [23]. Because the presence of these virus-related oncoprotein may affect the effect of BS69, we confirmed direct effect of BS69 siRNAs on LMP1-induced NF-κB activation in E7 negative human hepatoma Hep3B cells. As shown in Fig. 2C, siRNA-mediated reduced expression of BS69 resulted in a significant enhancement of LMP1-induced NF-κB-LUC activities in the virus-related oncoprotein negative cells.

NF-κB activation induces the production of inflammatory cytokines, such as IL-6. To confirm the regulation of LMP1-induced NF-κB activation by BS69, LMP1-induced IL-6 mRNA expression was analyzed in the BS69 siRNA-treated HeLa cells. LMP1-induced IL-6 mRNA expression was augmented in LMP1-transfected HeLa cells in parallel with the decrease of BS69 (Fig. 2D). To further assess the effects of BS69 on

LMP1-induced canonical NF-κB activation, we examined the effects of BS69 siRNA on LMP1-induced IκBα degradation. The BS69 siRNA-mediated reduction of BS69 markedly enhanced LMP1-induced IκBα degradation in HeLa cells (Fig. 2E). In addition, IL-6 mRNA expression and IκBα degradation were slightly enhanced in the BS69 siRNA-treated HeLa cells even in the absence of LMP1-expression. There might be the possibility that endogenous BS69 regulates basal NF-κB activation by interacting with potent NF-κB activators such as TRAF6, because BS69 has been shown to bind to TRAF6 [11]. At the present time, we do not know the mechanisms of regulation of basal NF-κB activation by BS69. Further detailed study will be required to clarify this issue. Taking these results together, BS69 acts as a negative regulator in LMP1-mediated NF-κB activation.

3. 2. Intracellular localization of BS69, LMP1, and NF-κB

We examined cellular co-localization of BS69 and LMP1 *in vivo* using confocal microscopy. HeLa cells were transfected with FLAG-tagged LMP1 and HA-tagged BS69 constructs. BS69 was mainly localized in the nucleus (Fig. 3A). However, when co-expressed with LMP1, BS69 protein was in part concentrated in punctate vesicular-like and perinuclear structures where LMP1 was also mainly localized, demonstrating

that LMP1 co-localized with BS69 *in vivo* (Fig.3B). We further examined whether expression of BS69 has any effect of cellular localization of NF-κB p65 *in vivo*. As shown in Fig. 3C, even in the presence of BS69, NF-κB p65 protein was mainly localized in the cytoplasm. Co-localization of BS69 with NF-κB p65 and a significant nuclear translocation of NF-κB p65 were not observed, suggesting that BS69 has no direct effect on NF-κB p65 in the regulation of NF-κB signaling.

3. 3. BS69 regulates LMP1-induced NF-κB activation by displacing TRADD from LMP1

In a previous study, BS69 has been demonstrated to interact with the CTAR2 domain of LMP1 and activate the JNK pathway by interacting with TRAF6 [11]. To further examine the molecular mechanisms of BS69-mediated suppression of NF-κB activation, we tested whether BS69 associates with other downstream molecules through the CTAR2 domain for LMP1-mediated NF-κB activation such as TRADD and STAP-2. We have demonstrated that siRNA-mediated reduction of TRADD results in a significant decrease of LMP1-mediated NF-κB-LUC activities in HeLa cells, whereas siRNA-mediated reduction of STAP-2 revealed a significant enhancement of NF-κB activation in HeLa cells [19]. To delineate the molecular interactions between BS69 and

these molecules, we first examined whether BS69 directly interacts with TRADD or STAP-2 *in vivo*. 293T cells were transfected with either HA-tagged TRADD or Myc-tagged STAP-2 together with HA-tagged BS69. Western blot analysis of the associated proteins with an anti-HA or anti-Myc antibody revealed that BS69 did not interact with either STAP-2 or TRADD (Fig.4A & 4C). BRAM1 also failed to interact with STAP-2 or TRADD (Fig.4B & 4D). We previously demonstrated that STAP-2 displaces TRADD from LMP1 and inhibits LMP1-mediated NF-κB activation. We then examined the effects of BS69 or BRAM1on the interactions between LMP1 and TRADD. Interestingly, TRADD failed to bind to LMP1 in the presence of BS69 or BRAM1 (Fig. 4E & 4F), indicating that BS69 and BRAM1 inhibit physical interactions between LMP1 and TRADD. These results suggest that BS69 decreases the formation of LMP1/TRADD complexes through the C-terminal region encoding BRAM1.

3. 4. Concluding remarks

We here demonstrate that BS69 negatively regulates LMP1-mediated NF-κB activation by displacing TRADD from LMP1. Previous studies demonstrated that BS69 acts as a transcription repressor in association with a variety of transcription factors such as c-Myb, B-Myb, Ets2 and MGA [24-27]. BS69 has also been shown to repress

transcription by recruiting N-CoR [28]. Furthermore, BS69 has also been shown to associate with mitotic chromosomes and interacts with Brg1 (the catalytic subunit of the mammalian SWI/SNF complex), indicating another role of BS69 in chromatin remodeling [29]. BS69 was originally identified as an adenovirus E1A-binding protein that inhibits the transactivation function of E1A [16] by interacting with the PXLXP motif present in E1A. These functions may be coincident with the fact that BS69 negatively regulates EBV LMP1-mediated NF-κB and JNK signaling pathways. Interestingly, BRAM1, an alternatively spliced form of BS69, retains the full MYND domain and is shown to interact with the BMP receptor 1A. Recently, BRAM1 has been shown to associate with the C-terminal domain of LMP1 through its MYND domain. Interactions between LMP1 and BRAM1 result in the inhibition of LMP1-mediated NF-κB activation but not JNK activation. BRAM1 influences LMP1-mediated and TNF-α-induced NF-κB activation by targeting the posttranslational modifications of IκB. In the present study, we show another possible mechanism that BS69 as well as BRAM1 can displace TRADD from LMP1 and inhibit LMP1- mediated NF-κB activation. We also tested whether BS69 and BRAM1 bind to LMP1 ID mutant, a site-specific mutant of LMP1 with a defective TRADD binding site by a substitution of ID for YYD in the last three amino acid residues of the C-terminal region of LMP1 protein (positions 384 to

386) [10]. Both BS69 and BRAM1, but not TRADD interacted with LMP1 ID mutant protein (data not shown), suggesting that the BS69/BRAM1 binding site may be close but not identical to the TRADD binding site. The TRADD binding to LMP1 might be competed by BS69/BRAM1 due to their steric effects. More detailed analysis of molecular interactions between LMP1, TRADD and BS69/BRAM1 will be required.

BS69 has also been demonstrated to interact with EBV-encoded latency protein EBNA2 [30]. EBNA2 is a nuclear transcriptional activator that is essential for EBV-induced cellular transformation. BS69 may also regulate EBNA2-mediated transcriptional activation in the nucleus of the EBV-infected cells. Interestingly, we previously showed that LMP1 expression upregulated STAP-2 expression, suggesting that STAP-2 plays a role in the defense of host cells against EBV infection. However, we could not observe any alteration of BS69 mRNA expression by LMP1 expression (data not shown). This finding indicates that BS69 is constitutively expressed and functions in the defense of host cells against EBV infection by interfering with dysregulated transcriptional activation mediated by LMP1 or EBNA2. We have also provided evidence that BS69 can inhibit EBV LMP1-mediated NF-□B activation through a direct modulation of interactions with TRADD, and our proposed mechanisms are now illustrated in Fig. 5. Our data suggest the possibility that BS69

may be a novel candidate for anti-viral therapy to regulate EBV LMP1-induced NF-κB and JNK pathways.

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Figure legends

Fig. 1. BS69 negatively regulates LMP1-induced NF-κB activation.

(A) HeLa cells in 12-well plates were transfected with HA-tagged LMP1 (10 ng) and NF-κB-LUC (100 ng) and/or increasing amounts of HA-tagged BS69 (30, 150 or 300 ng) using jetPEI. At 48 h after transfection, the cells were harvested and assayed for their luciferase activities using a Dual-Luciferase Reporter Assay System. An aliquot of each total cell lysate (TCL) was analyzed by immunoblotting with an anti-HA antibody. (B) 293T cells in 12-well plates were transfected with HA-tagged LMP1 (10 ng) and NF-κB-LUC (100 ng) and/or increasing amounts of empty vector, HA-tagged BS69 or BRAM1 (150, 300 ng). At 48 h after transfection, the cells were harvested and the luciferase activities were measured. An aliquot of each TCL was analyzed by immunoblotting with an anti-HA antibody. (C) 293T cells in 12-well plates were transfected with HA-tagged LMP1 (10 ng) and NF-κB-LUC (100 ng) and/or increasing amounts of FLAG-tagged TRAF1 (150, 300 ng). At 48 h after transfection, the cells were harvested and the luciferase activities were measured. An aliquot of each TCL was analyzed by immunoblotting with an anti-FLAG antibody. (D) HeLa cells in 12-well plates were transfected with HA-tagged LMP1 (10 ng) and/or increasing amounts of

empty vector, HA-tagged BS69 or BRAM1 (150 or 300 ng). At 36 h after transfection, the cells were stimulated with TNF-α (10 ng/ml) for additional 8 h. The stimulated cells were harvested, and luciferase activities were measured. An aliquot of each TCL was analyzed by immunoblotting with an anti-HA antibody. (E) 293T cells in 12-well plates were transfected with HA-tagged LMP1 (10 ng) and/or increasing amounts of empty vector, HA-tagged BS69 or BRAM1 (150, 300 ng). At 48 h after transfection, the cells were lysed and total extracts were immunoblotted with anti-pJNK, anti-JNK, anti-HA or anti-actin antibodies.

Fig. 2. Reduction of endogenous BS69 enhances LMP1-induced NF-κB activation. (A) HeLa cells in 24-well plates were transfected with a control siRNA or siRNAs targeting human BS69 (#1 and #2). The BS69 expression levels were quantified by RT and quantitative real-time PCR analysis. Data represent the levels of BS69 mRNA normalized by that of G3PDH mRNA as an internal control and are expressed relative to the value at time zero. Data represent the means of duplicate PCR determinations, which generally varied by <10%. (B) HeLa cells in 24-well plates were transfected with a control siRNA or BS69 siRNAs (#1 and #2). The cells were then transfected with HA-tagged LMP1 (3 ng) and NF-κB-LUC (100 ng) using jetPEI. At 48 h after

transfection, the cells were harvested and the luciferase activities were measured. At least three independent experiments were carried out for each assay. *$p<0.05$ **$p<0.01$. An aliquot of each total cell lysate (TCL) was analyzed by immunoblotting with an anti-HA antibody. (C) Hep3B cells in 24-well plates were transfected with a control or BS69 siRNA (#2). The cells were then transfected with HA-tagged LMP1 (3 ng) and NF-□B-LUC (100 ng) using jetPEI. At 48 h after transfection, the cells were harvested and the luciferase activities were measured. At least three independent experiments were carried out for each assay. *$p<0.01$. An aliquot of each TCL was analyzed by immunoblotting with an anti-HA antibody. (D) HeLa cells in 12-well plates were transfected with a control siRNA or BS69 siRNAs (#1 and #2). The cells were then transfected with HA-tagged LMP1 (1.5 □g) using jetPEI. At 48 h after transfection, total RNA samples were extracted and analyzed for their IL-6 mRNA expression levels by RT and quantitative real-time PCR analysis. Data represent the levels of IL-6 mRNA normalized by that of G3PDH mRNA as an internal control and are expressed relative to control siRNA-treated samples without LMP1. Data represent the means of duplicate PCR determinations, which generally varied by <10%. An aliquot of each TCL was analyzed by immunoblotting with an anti-HA antibody. (E) HeLa cells in 12-well plates were transfected with a control siRNA or BS69 siRNAs (#1 and #2). The cells were

then transfected with HA-tagged LMP1 (1.5 μg) using jetPEI. At 48 h after transfection, the cells were lysed and total extracts were immunoblotted with anti-IκBα, anti-HA or anti-actin antibodies.

Figure 3. BS69 co-localizes with LMP1.

(A)HeLa cells in a 12-well plate were transfected with FLAG-tagged LMP1 (1 μg) or HA-tagged BS69 (1 μg) using jetPEI. At 48 h after transfection, the cells were fixed and reacted with anti-FLAG or anti-HA antibodies, and visualized with FITC- or rhodamine-conjugated secondary antibody. The same slide was also stained with DAPI for the nuclei staining. These figures were merged. (B) HeLa cells in a 12-well plate were transfected with FLAG-tagged LMP1 (1 μg) and HA-tagged BS69 (1 μg) using jetPEI. At 48 h after transfection, the cells were fixed and reacted with anti-FLAG and anti-HA antibodies, and visualized with FITC- or rhodamine-conjugated secondary antibody. These figures were merged. The same slide was also stained with DAPI for the nuclei staining. The arrowheads show co-localization of BS69 with LMP1. (C) HeLa cells in a 12-well plate were transfected with HA-tagged BS69 (1 μg) using jetPEI. At 48 h after transfection, the cells were fixed and reacted with anti-NF-κB p65 and anti-HA antibodies, and visualized with FITC- or rhodamine-conjugated

secondary antibody. These figures were merged. The same slide was also stained with DAPI for the nuclei staining.

Fig. 4. BS69 displaces TRADD from LMP1

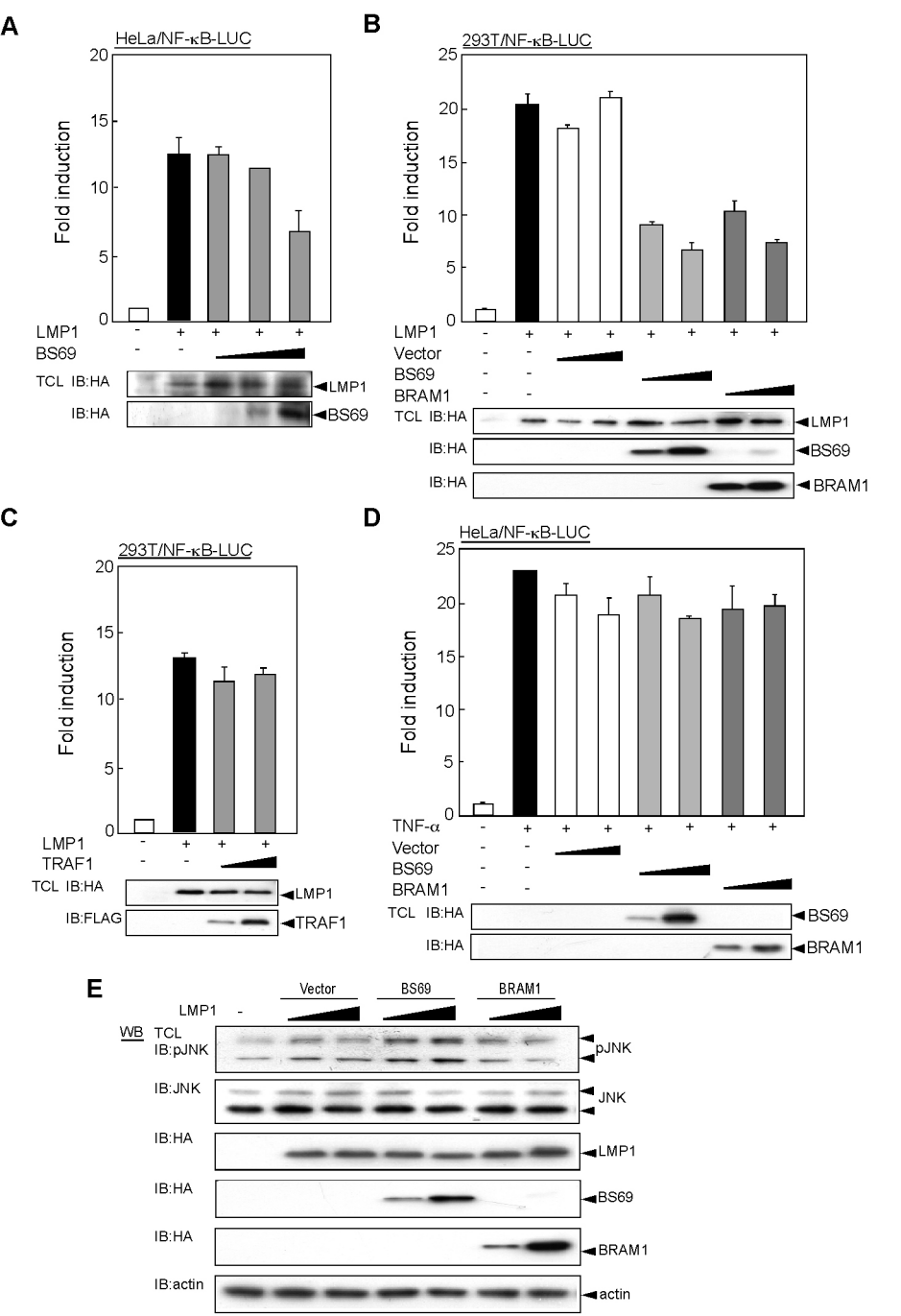
(A) 293T cells ($1x10^7$) were transfected with Myc-tagged STAP-2 (10 μg) with or without HA-tagged BS69 (10 μg). At 48 h after transfection, the cells were lysed, immunoprecipitated with an anti-HA antibody and immunoblotted with an anti-Myc or anti-HA antibody. An aliquot of each total cell lysate (TCL) was immunoblotted with the anti-Myc antibody. (B) 293T cells ($1x10^7$) were transfected with Myc-tagged STAP-2 (10 μg) with or without HA-tagged BRAM1 (10 μg). At 48 h after transfection, the cells were lysed, immunoprecipitated with an anti-HA antibody and immunoblotted with an anti-Myc or anti-HA antibody. An aliquot of each TCL was immunoblotted with the anti-Myc antibody. (C) 293T cells ($1x10^7$) were transfected with HA-tagged TRADD (10 μg) with or without FLAG-tagged BS69 (10 μg). At 48 h after transfection, the cells were lysed, immunoprecipitated with an anti-FLAG antibody and immunoblotted with an anti-HA or anti-FLAG antibody. An aliquot of each TCL was immunoblotted with the anti-HA antibody. (D) 293T cells ($1x10^7$) were transfected with HA-tagged TRADD (10 μg) with or without HA-tagged BRAM1 (10 μg). At 48 h

after transfection, the cells were lysed, immunoprecipitated with an anti-TRADD antibody and immunoblotted with an anti-HA antibody. An aliquot of each TCL was immunoblotted with the anti-HA antibody. (E) 293T cells ($1x10^7$) were transfected with FLAG-tagged LMP1 (10 □g) or HA-tagged TRADD (10 □g) and/or HA-tagged BS69 (10 □g). At 48 h after transfection, the cells were lysed, immunoprecipitated with anti-FLAG antibody and immunoblotted with an anti-HA or anti-FLAG antibody. An aliquot of each TCL was immunoblotted with the anti-HA antibody. (F) 293T cells ($1x10^7$) were transfected with FLAG-tagged LMP1 (10 □g) or HA-tagged TRADD (10 □g) and/or HA-tagged BRAM1 (10 □g). At 48 h after transfection, the cells were lysed, immunoprecipitated with anti-FLAG antibody and immunoblotted with an anti-HA or anti-FLAG antibody. An aliquot of each TCL was immunoblotted with the anti-HA antibody.

Fig. 5. Scheme of functional proposal of BS69 in the suppression of LMP1-induced NF-□B activation

LMP1 consists N-terminal tail, six transmembrane domain and a long cytoplasmic C-terminal domain which contains two NF-□B activating domain, CTAR1and CTAR2. BS69 directly interacts with LMP1 and suppresses LMP1-induced NF-□B activation

through CTAR2. BS69 and its alternative spliced form, BRAM1 regulate CTAR2-mediated canonical NF-κB activation by displacing TRADD from LMP1. LMP1-induced JNK activation is mediated by BS69 and TRAF6. BS69 also interacts with EBV-encoded latency nuclear protein EBNA2.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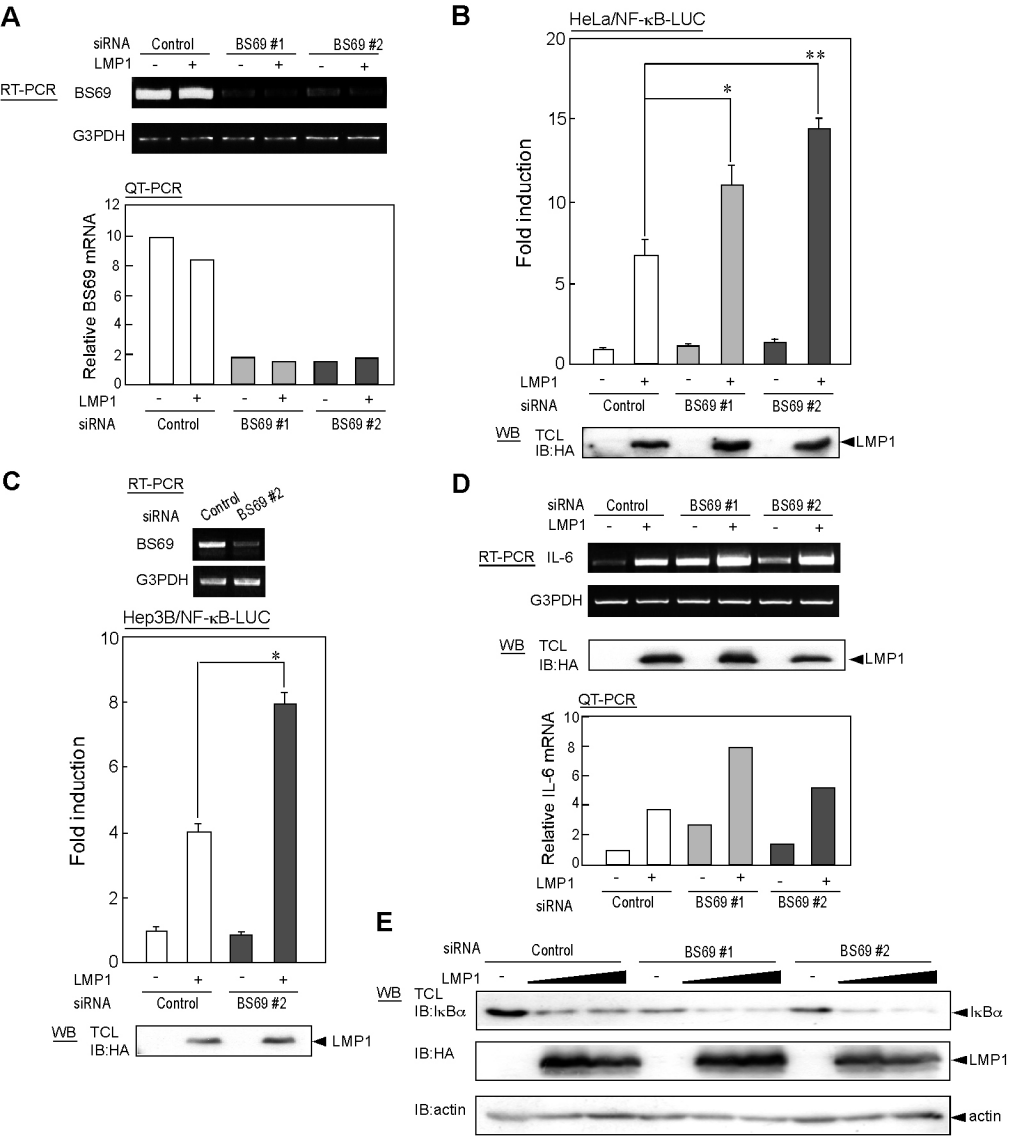
actin

Figure 1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Fold induction
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TCL
IB:HA
LMP1
C
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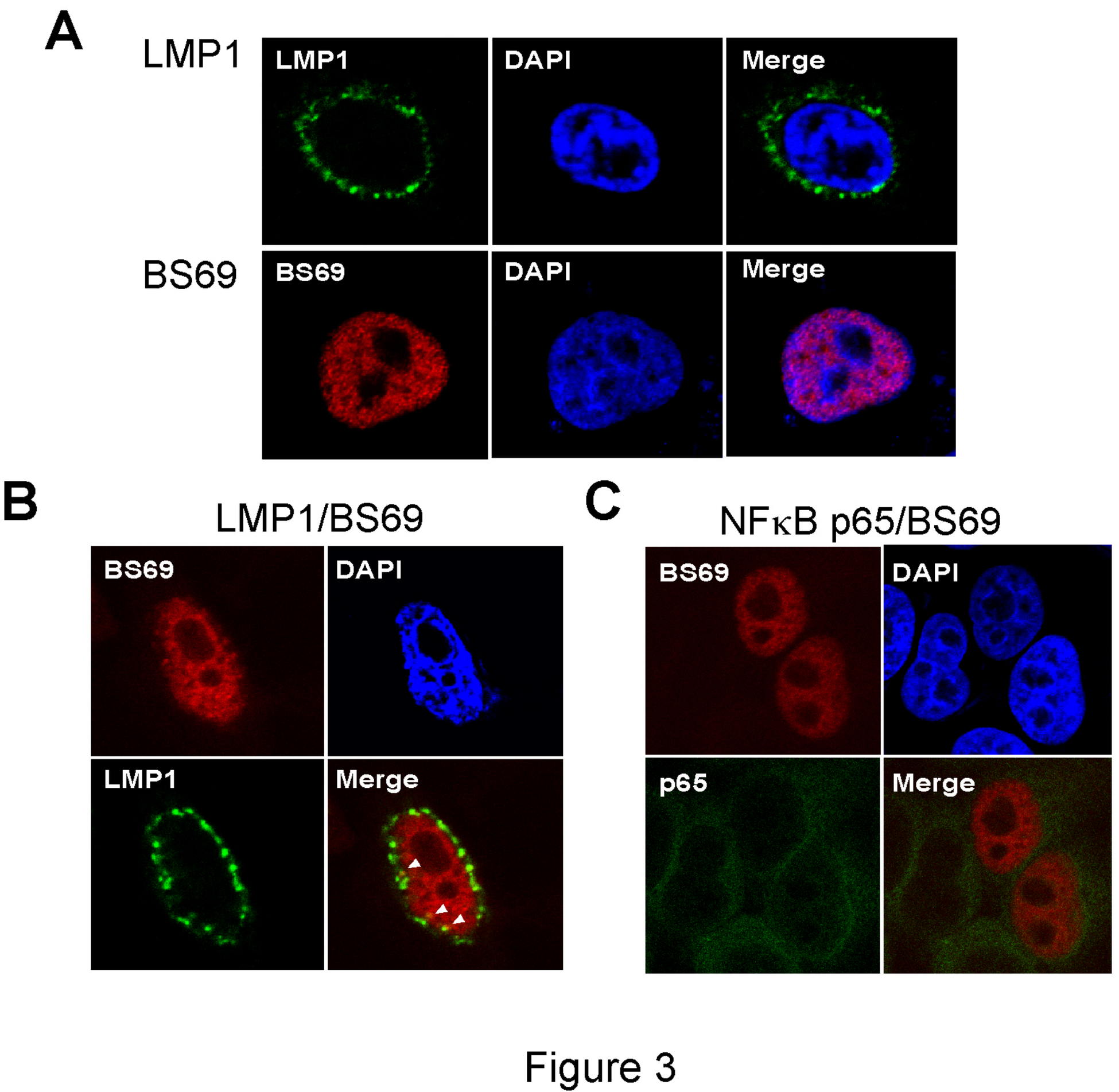
Figure 2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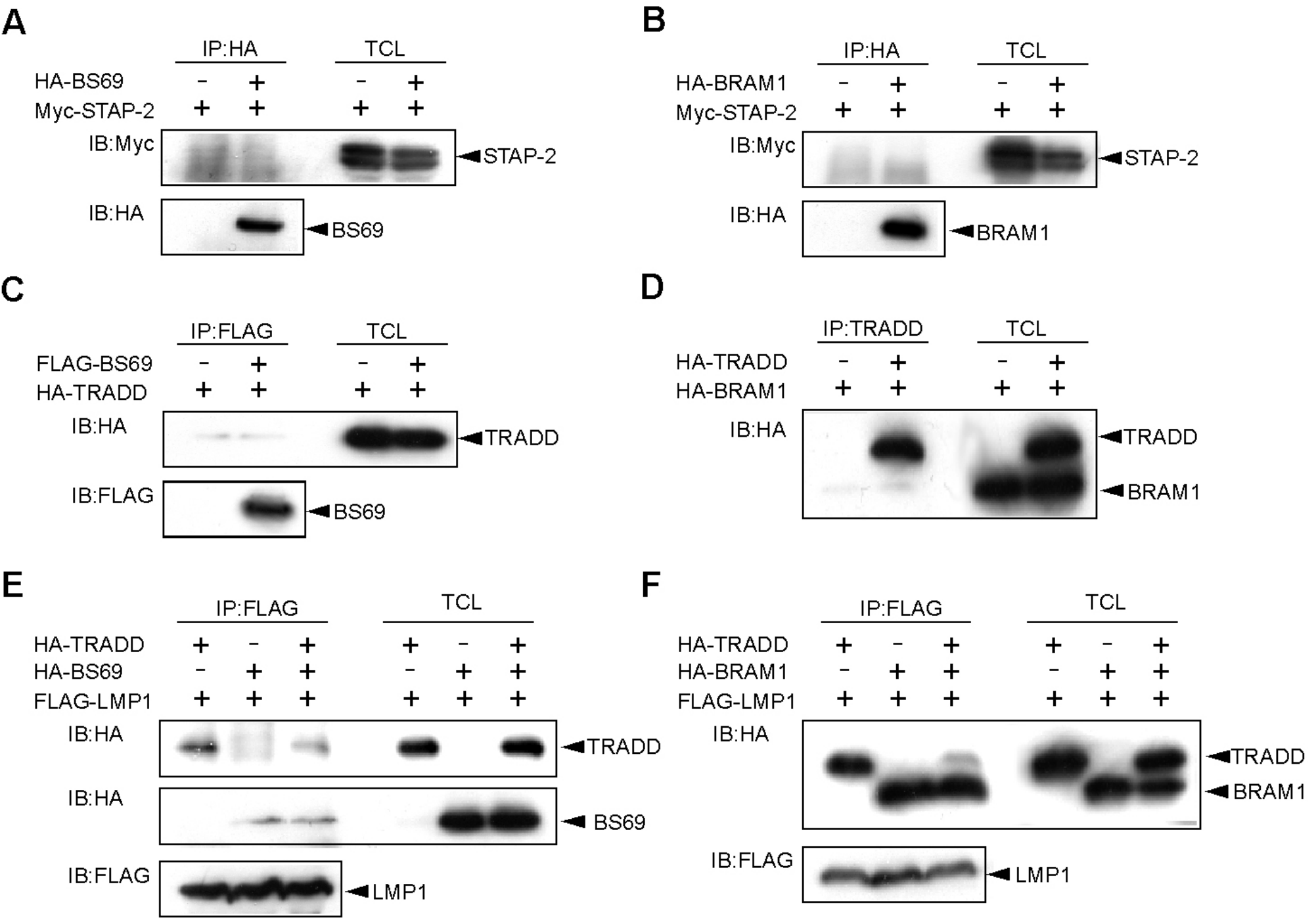
NFκB p65/BS69
BS69
DAPI
p65
Merge

Figure 3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C
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TCL
FLAG-BS69
HA-TRADD
IB:HA
TRADD
IB:FLAG
BS69
D
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TCL
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BS69
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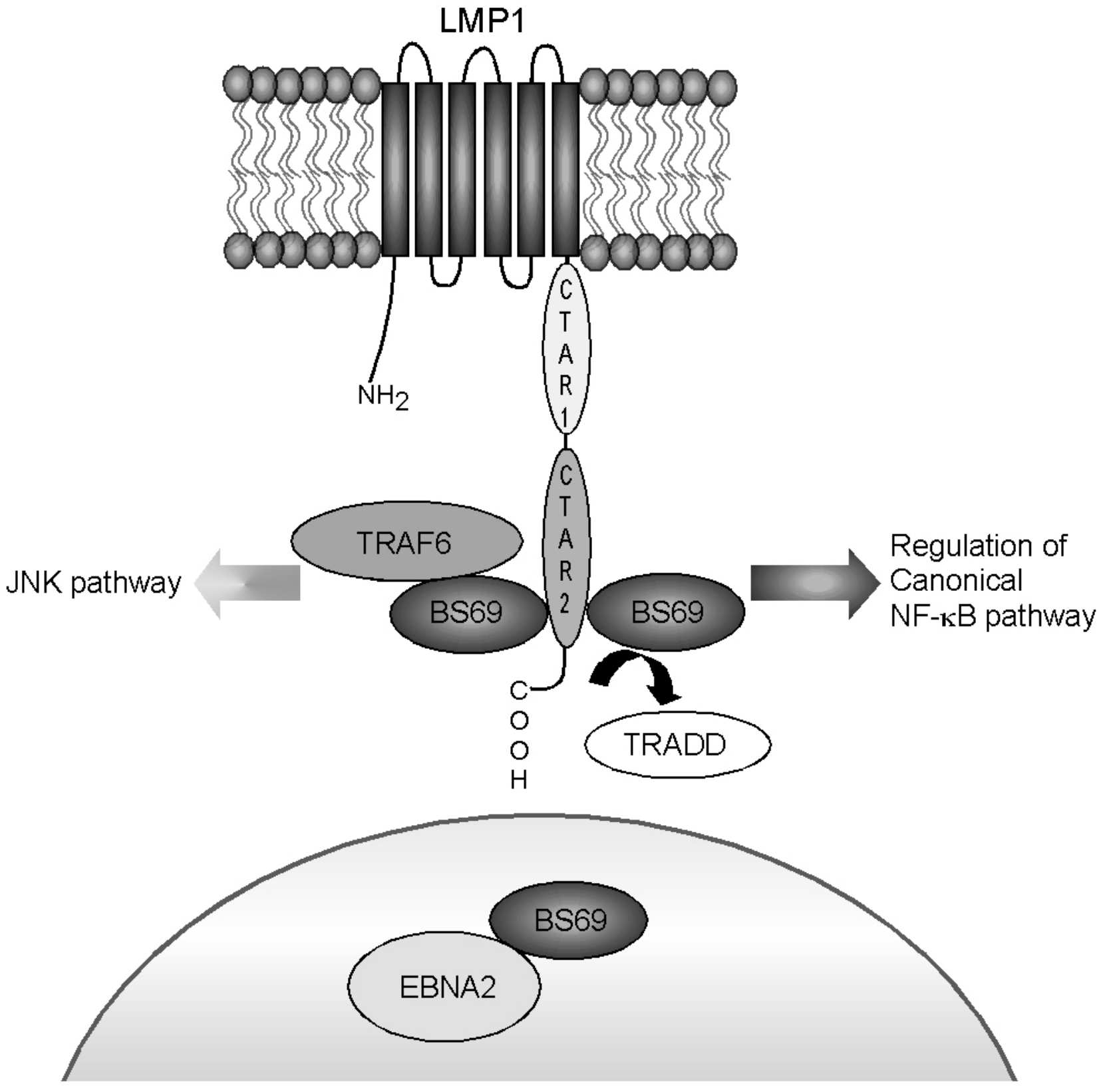
Figure 4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CTAR2
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JNK pathway
BS69
BS69
Regulation of
Canonical
NF-κB pathway
COOH
TRADD
BS69
EBNA2

Figure 5