Nuclear Import of Epstein-Barr virus BLLF2 is Mediated by an Importin β1-Dependent Mechanism

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Research

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Abstract

**Background:** Epstein-Barr virus (EBV), the pathogen of several human malignancies, encodes many proteins that require to be transported into the nucleus for viral DNA reproduction and nucleocapsids assembly in the lytic replication cycle. A nuclear membrane phosphoprotein encoded by EBV *BLLF2*, is believed to associate with viral DNA packaging and primary egress across the nuclear membrane.

**Results:** Here, fluorescence microscope, mutation analysis, interspecies heterokaryon assays, co-immunoprecipitation assays and western blot were performed to explore the nuclear import mechanism of BLLF2. As results, BLLF2 was shown to be a nucleocytoplasmic shuttling protein, which was mediated neither by chromosomal region maintenance 1 (CRM1)- nor transporter associated with antigen processing (TAP)-dependent pathway. Yet, two functional nuclear localization signals (NLSs) of BLLF2, NLS1 (16KRQALETVPHPQNRGR31) and NLS2 (48PPVAKRRR58), were identified, whereas the predicted NES was nonfunctional. Finally, BLLF2 was proved to transport into the nucleus via Ran-dependent and importin β1-dependent pathway.

**Conclusions:** This mechanism may contribute to a more extensive insight of the assembly and synthesis of EB virions in the nucleus, thus affording a new direction for the treatment of viruses.

Background

Epstein-Barr virus (EBV), a member of gamma herpesvirus subfamily, is the most universal and persistent pathogen prevails in humanity, with almost 90% of the world’s population maintaining a subclinical lifelong infection [1]. As the first discovered human tumor virus, EBV is related to several human malignancies, including infectious mononucleosis, nasopharyngeal carcinoma, Burkitt lymphoma, diverse lymphoproliferative disorders and endemic Hodgkin lymphoma [2]. Despite EBV holds a latent infection in the host cells, it can intermittently exchange from the latent phase to lytic cycle, followed by the induction of more than 80 viral constituents, the synthesis of viral genomic DNA, and eventually the production of progeny virions [3]. Upon stimulation, the viral immediate-early transcriptional activators Rta and Zta are firstly expressed to enhance the expression of early genes, which include those are essential for viral DNA genome replication. After viral DNA amplification in the replication focuses of the nucleus, viral late gene transcription takes place, this process encodes diverse categories of viral structural components, such as capsid proteins, tegument proteins and glycoproteins, which are vital for DNA replication, viral morphogenesis or virion composition [3]. In lytic phase, a number of EBV-encoded proteins are transported into the nucleus for viral DNA replication and nucleocapsid assembly.

The trafficking of target proteins between the nucleus and cytoplasm of eukaryote is achieved by means of the nuclear pore complex (NPC) embedded in the nuclear membrane, which bears a extremely preserved architecture with an eightfold rotational symmetry that carries a central aqueous cylindrical tunnel embraced with a huge number of specialized appendages [4]. Dissimilar with small molecule that move into and out of the nucleus on the way of simple diffusion, the nucleocytoplasmic shuttling of large
molecule is accomplished by various cellular transporter exportins and importins, by distinguishing favourable motifs on the target proteins named nuclear localization signal (NLS) and nuclear export signal (NES) [4].

Nuclear trafficking of a definitive protein is often achieved by the canonical importin α/β-reliant nuclear translocation pathway. As an adaptor protein, importin α binds to target protein encompassing with NLS and then heterodimerizes with importin β, to assemble the heterotrimeric importin α/β/NLS-cargo complex that penetrates through NPC and delivers NLS-cargo into the nucleus [5, 6]. Additionally, another crucial component of the nuclear import pathway, Ras-related nuclear protein (Ran), is an eukaryotic evolutionarily preserved small GTPase. Both effective export and import are regulated by a gradient of Ran in GTP- and GDP-combined states between the cytoplasm and the nucleus. After attaching with RanGTP, the nuclear transport receptors binding cargo can be trafficked from the cytoplasm to the nucleus, while export receptors can release goods via binding to RanGTP and give off them from the nucleus into the cytoplasm after GTP hydrolysis [5].

Nuclear export of proteins is largely fulfilled by a leucine-rich NES, which is bound by the major nuclear export receptor chromosomal region maintenance 1 (CRM1) (exportin 1, XPO1) that belongs to the the karyopherin-β family. Ttransporter associated with antigen processing (TAP/NXF1) is also a major export receptor, both of them export mRNA from the nucleus to cytoplasm in metazoan cells have been profoundly investigated. Despite TAP is not a strong RNA-binding protein, it mainly binds to the Aly/REF mRNA adaptor protein, an element of the messenger ribonucleoprotein particles (mRNPs), which is carried out of the nucleus via direct combination with nucleoporins imbedding into the nuclear pore [7]. TAP/NXF1 also can improve the nuclear export of some proteins [8–10], whereas CRM1 can export hundreds of cargo proteins out of the nucleus, by combining to their classical leucine-rich NESs [11]. The CRM1-relied export pathway is widely utilized to export proteins and non-coding RNAs, including ribosomal RNAs (rRNAs) and small nuclear RNAs (snRNAs), while only a minority of cellular mRNAs employ this pathway [11, 12]. In addition to CRM1 and TAP, other exportins are also involved in the nuclear export process. Exportin 4 is in charge of the nuclear exports of eukaryotic mothers against decapentaplegic homolog 3 (Smad3) and translation initiation factor 5A (eIF5A) [13]. Exportin 5 can mediate the export of dsRNA and precursor microRNA, while exportin-t can help the export of tRNA [14–17]. Importantly, exportin cellular apoptosis susceptibility protein (CAS) can assist the export of importin α for a new cycle of protein nuclear translocation [18]. Besides, exportin 7 is a nuclear export mediator with broad substrate specificity [19].

It's well documented that the nuclear accumulation of herpesviral proteins is significant for virus propagation, assembly and dissemination, whereas the nuclear transport mechanisms of the majority of virus-encoded proteins are less well explored. As a transcriptional co-activator of EBV nuclear antigen 2 (EBNA2), EBNA-LP interplays with importin α1 through functional NLS, to facilitate its efficient nuclear localization for its interaction with EBNA2 [20]. The N-terminus functional NES of EBV early protein EB2 (also designated BMLF1, SM or Mta) can advance the nucleocytoplasmic export of a number of early and late viral mRNAs (come from intronless genes) by precisely binding to TAP/NXF1, which is indispensable
EBNA1 can interact with importin α1 and importin α5 via its NLS, to accelerate its nuclear import [6], which may be fundamental for the sustainment, proliferation and transcription of the EBV-positive tumor cells. BFLF2 is exhibited to be correlated with TAP for nuclear export, and interplayed with importin α7, importin β1 and transportin-1 for its nuclear accumulation, which may be meaningful for the efficient viral DNA packaging and primary release across the nuclear membrane. Furthermore, BGLF4 protein kinase can promote the nuclear accumulation of a few non-NLS-containing EBV proteins, including major capsid protein (VCA) and the viral DNA replication enzymes BBLF2/3, BBLF4 and BSLF1 [22]. However, the functional correlation of nucleocytoplasmic shuttling of most of the EBV proteins require to be probed.

EBV tegument protein BLLF2 is a nuclear membrane phosphoprotein, which may exert some roles in viral reduplication or virions assembling. Our previous study manifested that BLLF2 absolutely localizes in the nucleus [23], nonetheless the definite mechanism for its subcellular localization was not well known. In our preliminary experiment, we established that BLLF2 could shuttle between the cytoplasm and nucleus, which propels us to investigate the nucleocytoplasmic transport mechanism of BLLF2.

**Results**

**Nucleocytoplasmic shuttling of BLLF2**

Our previous study revealed that BLLF2 is definitely located in the nucleus [23]. To dissect the subcellular transport mechanism of BLLF2, bioinformatics analysis was initially implemented and showed that BLLF2 possesses four predicted potential NLS motifs (pat4 $^{44}$RRPR$^{47}$ and $^{52}$KRRR$^{55}$, and pat7 $^{48}$PPVAKRR$^{54}$ and $^{49}$PVAKRRR$^{55}$) and two supposed NES, that is NES1 ($^{83}$VQSPPQITAVIQL$^{95}$) and NES2 ($^{102}$MRPPIYL$^{108}$) (Fig. 1A). Subsequently, BLLF2 was again confirmed completely located in the nucleus in COS-7 cells transfected with EYFP-BLLF2 plasmid (Fig. 1B). In order to exclude the influence of the big tag EYFP on the subcellular localization of BLLF2, BLLF2-EYFP and BLLF2-Myc expression plasmids were also constructed, and fluorescence microscope demonstrated that the subcellular localization patterns of BLLF2-EYFP and BLLF2-Myc were similar with that of EYFP-BLLF2 (Fig. 1C). Besides, co-expression of nucleolar marker pNucleolin-EGFP with pEYFP-BLLF2 showed that BLLF2 also located in the nucleolus (Fig. 1D).

A nuclear localization protein equipped with functional NES and NLS can be theoretically transported from a donor to a recipient nucleus [24]. With regard to the heterokaryon assays, the shuttling characteristic of a certain protein can be established through the species-specific DAPI staining fashions of the nucleus under fluorescence microscope, with speckles in the nucleus of mouse NIH3T3 cells (Fig. 1E) [8, 25]. Since BLLF2 holds predicted NES and NLS, we wondered if BLLF2 can shuttle between the cytoplasm and nucleus. As results (Fig. 1F), COS-7 cells expressing EYFP-BLLF2 were fused with a considerable number of NIH3T3 cells in the presence of protein synthesis inhibitor cycloheximide. After fusing, BLLF2 could also be detected with a distinguishing speckled fashion in the nucleus of NIH3T3.
cells. As the nucleocytoplasmic shuttling positive control [25], EYFP-BFLF2 could shuttle between COS-7 cells and NIH3T3 cells, suggesting BLLF2 is a genuine nucleocytoplasmic shuttling protein.

Identification of the functional NES in BLLF2

On the basis of the predicted motifs of NESs and NLSs, full-length of BLLF2 was firstly cut into two fragments (amino acids (aa)1-58 and aa59-148) and then fused to the C-terminus of EYFP (Fig. 2A), to detect their subcellular localizations. As shown in Fig. 2B, aa1-58 was perfectly located in the nucleus and nucleolus, while aa59-148 displayed disperse dissemination throughout the cytoplasm and nucleus. These results disclosed that aa1-58 contains functional NLS, and aa59-148 may not contain functional NES.

To further find out the functional NES of BLLF2, pEYFP-BLLF2(83-148) was constructed (Fig. 2A) and transfected into COS-7 cells, which had the similar subcellular localization pattern with that of aa59-148 (Fig. 2B). Next, aa83-148 was shortened to aa83-110 (contains predicted NES1 and NES2) to create pEYFP-BLLF2(83-110) (Fig. 2A), and result uncovered that aa83-110 also homogeneously localized throughout the cytoplasm and nucleus (Fig. 2C). These results confirmed that the predicted NES1 and NES2 are nonfunctional.

Identification of the functional NLS in BLLF2

To analyze whether the predicted pat4 (44RRPR47) is a functional NLS, pEYFP-BLLF2(1-47) and pEYFP-BLLF2(1-43) were constructed (Fig. 2A) and tested in COS-7 cells. As results, aa1-47 was entirely located in the nucleus and nucleolus (Fig. 2D), indicating 44RRPR47 may take a effect on the nucleolus localization, or aa1-43 contains functional NLS. As expected, aa1-43 was also located in the nucleus without nucleolus (Fig. 2D), proving aa1-43 contains functional NLS, which maybe composed by 25HPQNRGRMLSPKARPPK41.

For the sake of ascertaining whether the predicted pat4 (52KRRR55) and pat7 (48PPVAKRR54 and 49PVAKRR55) in aa48-58 of BLLF2 are functional, pEYFP-BLLF2(48-82), pEYFP-BLLF2(48-58) and pEYFP-BLLF2(59-82) were constructed (Fig. 2A) and assessed in COS-7 cells. As shown in Fig. 2E, aa48-82 was predominantly located in the nucleus without nucleolus, demonstrating 48PPVAKRR55 may has a nuclear localization effect for aa48-82. Moreover, aa48-58 was mostly located in the nucleus, indicating aa48-58 can function as a NLS. However, aa59-82 showed a pan-cellular localization pattern, suggesting this region does not contain functional NLS.

To continue validate whether pat4 (44RRPR47) of aa44-58 has a role of nucleolus localization, pEYFP-BLLF2(44-58) was constructed (Fig. 2A) and examined in COS-7 cells. Result showed that aa44-58 was located in the nucleus and nucleolus (Fig. 2F), suggesting pat4 (44RRPR47) may be a functional nucleolus localization signal, and 44RRPRPPVAKRR58 is a authentic NLS. To convincingly certify the nucleolus localization role of 44RRPR47, 44RRPR47 was mutated to 44AAAA47 in the full length of BLLF2 and then
fused to the C-terminus of EYFP, to yield pEYFP-BLLF2(44-47)m. As a result, the nuclear and nucleolus localization pattern of BLLF2 was alternated into pan-nuclear localization without nucleolus (Fig. 2F). These data confirmed that $^{44}$RRPR$^{47}$ has nucleolus localization function.

Next, to further inspect the functional NLS of aa1-43, pEYFP-BLLF2(1-20) and pEYFP-BLLF2(21-43) were constructed (Fig. 3A) and assayed in COS-7 cells. As shown in Fig. 3B, both fluorescences of aa1-20 and aa21-43 had comparable subcellular localization pattern with that of the EYFP control, indicating these two regions do not contain functional NLS. Furthermore, when aa1-20 was extended to aa1-31, aa1-31 showed conspicuous nuclear localization (Fig. 3C), suggesting aa1-31 possesses functional NLS, which may be located in aa6-31 or aa16-31.

To eventually determine the minimum NLS region of aa1-43, pEYFP-BLLF2(6-31) and pEYFP-BLLF2(16-31) were constructed (Fig. 3A) and transfected into COS-7 cells. As results, aa6-31 and aa16-31 showed parallel subcellular localization to that of aa1-31, with dominant nuclear localization (Fig. 3D), disclosing aa16-31 is another functional NLS of BLLF2.

Nuclear import mechanism of BLLF2

Ran (Ras-associated nuclear protein), a small GTPase belonging to the RAS superfamily, is specialized and crucial for the nuclear accumulation of proteins with a canonical NLS [26]. Here, dominant negative (DN) RanGTP containing Q69 mutation (Ran-Q69L), without the competence of GTP hydrolysis [8], was applied to dissect whether Ran is vital for the nuclear trafficking of BLLF2. Compared to the cells co-transfected with mCherry vector and pEYFP-BLLF2, the nuclear import of BLLF2 was undoubtedly confined in cells co-expressing Ran-Q69L-mCherry and EYFP-BLLF2 (Fig. 4B), suggesting the nuclear translocation of BLLF2 is Ran-relied and requires Ran GTP hydrolysis.

It’s universally established that the importin α/β heterodimer can discern canonical NLS and facilitate the nucleocytoplasmic transport of particular target proteins [27]. In order to resolve which receptor takes part in the nuclear translocation of BLLF2, DN importin α5 (κα1, flawed in the binding to importin β [28]), as well as DN importin β1 (κβ1, faulty in associating with Ran [28]), were exploited. Moreover, the nuclear import inhibitors of M9M (specifically impedes importin β2 to attach to NLS) and Bimax2 (suppresses the functions of importin α1, α3 and α7 [8, 25]) were also applied. Compared to the negative controls of DNα or competitive inhibitors (Fig. 4A), BLLF2 was relocalized to the cytoplasm by DN κβ1, but not DN κα1, M9M, Bimax2 or mCherry (Fig. 4B), suggesting BLLF2 may be transported into the nucleus via Ran- and importin β1-dependent pathway.

Nuclear export mechanism of BLLF2

CRM1 (Exportin1/XPO1), a member of the importin β family, mediates the nuclear export of proteins by combining to their classical NESs [11]. Therefore, we go on to assess whether the nuclear export of BLLF2 can be blocked by CRM1 specific inhibitor leptomycin B (LMB) [8, 25]. As CRM1-dependent positive control [29], the nuclear accumulation of UL4 was inhibited by LMB treatment (Fig. 5A), whereas
the CRM1-independent negative control of EYFP vector was incapable to achieve the nucleocytoplasmic shuttling with or without the presence of LMB (Fig. 5B). Upon LMB treatment, EYFP-BLLF2 was also transported from monkey nuclei to mouse nuclei (Fig. 5C), indicating BLLF2 has the capability to shuttle between varied cells, and the nuclear export of BLLF2 may take place independently on CRM1.

It is reported that CRM1 overexpression can advance the nuclear export of CRM1-dependent proteins [25]. As results, the nuclear export of the CRM1-dependent positive control HSV-1 UL4 [29] was promoted by co-expression of CRM1-mCherry, yet BLLF2 remain thoroughly located in the nucleus and nucleolus in the existence of CRM1 when COS-7 cells were co-transfected with pCRM1-mCherry and pEYFP-BLLF2 (Fig. 5D), certifying CRM1 is not fundamental for the nuclear export of BLLF2.

Besides CRM1, TAP (NXF1), the critical mRNA export receptor, is also related to the nuclear export of distinct proteins [30], which also can expedite the nuclear export of TAP-relied protein when it is overexpressed with the target protein [25]. As shown in Fig. 5E, the nuclear export of the TAP-dependent positive control BFLF2 [25] was obviously boosted by co-expression of TAP-mCherry, whereas TAP could not transport BLLF2 from the nucleus to cytoplasm when COS-7 cells were co-transfected with EYFP-BLLF2 and TAP-mCherry expression plasmids, testifying the nuclear export of BLLF2 was also independent on TAP.

BLLF2 binds to importin β1

To finally validate the aforementioned assumption, the interactions of BLLF2 with human importin α/β molecules, importin α1 (κα2), importin α3 (κα4), importin α5 (κα1), importin α7 (κα6), importin β1 and importin β2, were inspected. Plasmids expressing EYFP-BLLF2, EYFP-BFLF2, PRV UL31-EYFP or EYFP vector was co-transfected with Flag-tagged importins or Flag vector into HEK293T cells for 24 h, then cell lysates were harvested for Co-IP assays. In comparison to the mouse negative IgG (Fig. 6), EYFP-BLLF2 was perfectly Co-IPed with 3×Flag-importin β1 (by using anti-Flag mAb) (Fig. 6A), rather than κα1 (Fig. 6B), κα2 (Fig. 6C), κα4 (Fig. 6D), κα6 (Fig. 6E) or importin β2 (Fig. 6F). In order to verify the interaction between EYFP-BLLF2 and 3×Flag-importin β1, reversed Co-IP was performed with anti-YFP antibody, and result showed that 3×Flag importin β1 could be effectively pulled down by EYFP-BLLF2 (Fig. 6G). As positive controls [25, 31], EYFP-BFLF2 and PRV UL31-EYFP could be Co-IPed with anti-Flag mAb when HEK293T cells were co-transfected with plasmids expressing EYFP-BFLF2/3×Flag-importin β1 (Fig. 6H) or PRV UL31-EYFP/3×Flag-importin β1 (Fig. 6I). However, no EYFP protein (Fig. 6J), 3×Flag-importin β1 (Fig. 6K) or EYFP-BLLF2 (Fig. 6L) was Co-IPed with anti-Flag mAb (Fig. 6J), anti-YFP pAb (Fig. 6K) or anti-Flag mAb (Fig. 6L) when HEK293T cells were co-transfected with EYFP vector and Flag vector (Fig. 6J), EYFP vector and 3×Flag-importin β1 (Fig. 6K) or EYFP-BLLF2 and Flag vector (Fig. 6L), proving BLLF2 can interplay with the nuclear import receptor importin β1, but not importin α1, α3, α5, α7 or importin β2.

Discussion

Subcellular distribution of nuclear protein was firstly disclosed to shuttle back and forth between the cytoplasm and nucleus by transplantation experiment in *Amoeba proteus* [32], and a increasing number
of proteins are proved to have the ability of nucleocytoplasmic shuttling [33]. Nonetheless, the nucleocytoplasmic shuttling feature of protein from higher eukaryotic cells is shown based on cell fusion experiment. In interspecies heterokaryon assays, we found that the phosphorylated nuclear membrane protein BLLF2, which may act a vital role for the viral proliferation or virions assembling, could locate in the nucleus and nucleolus, and shuttle between the cytoplasm and nucleus.

When herpesvirus invades cells, the tegument proteins are delivered into the cytoplasm, which may stay in the cytoplasm or transfer into the nucleus to achieve their corresponding functions, such as inhibiting the transcription and translation of the host cells, restraining the host innate immunity, etc. to facilitate the propagation of the virus. After replication, the virus will synthesize a series of structural proteins to assemble progeny virions, which also play different roles in the life cycle of the virus. In the case of transient transfection, the newly synthesized BLLF2 fusion protein will also locate in the specific cell compartments to perform its function(s), which can simulate the function(s) and/or characteristics of BLLF2 to a certain extent during EBV infection. As we known, specific antibody is the key tool to investigate the function of target protein. In our previous experiment design, we had considered we could use specific BLLF2 antibody to detect the time course expression and subcellular localization of BLLF2 during EBV lytic infection induced from EBV latent cells, which can be used to analyze the correlation between the function(s) and characteristics of BLLF2 during EBV lytic infection and BLLF2 transient expression. We had tried to induce the expression of BLLF2 in prokaryocyte (E.coli) to prepare the BLLF2 antibody by using the mature antibody preparation technology in our laboratory [34–37], but the expression of BLLF2 could not be effectively induced after many attempts. Thus, the BLLF2 antibody was not successfully prepared. Besides, there is no commercial BLLF2 antibody available, we therefore unable to analyze the correlation between the function(s) and characteristics of BLLF2 during EBV lytic infection and BLLF2 transient expression.

For accomplishing nucleocytoplasmic shuttling, nuclear localization of a specific protein demands the engagement of functional NES and NLS. Bioinformatics analysis revealed that BLLF2 carries four potential NLS motifs (pat4 44RRPR47 and 52KRRR55, and pat7 48PPVAKRR54 and 49PVAKRRR55) and two potential NES 83VQSPPQITAVIQL95 (NES1) and 102MRPPIYL108 (NES2). In the present study, aa1-82 was divided into two regions aa1-47 and aa48-82. In aa1-47, aa16-31 was identified as a functional NLS (NLS1). Moreover, aa48-58 was also established as a functional NLS (NLS2) in aa48-82. Since these two functional NLSs are not adjacent to each other, and the functional NLS is generally not long. Thus, the functional NLSs of BLLF2 are aa16-31 and aa48-58. Besides, aa44-47 exerts a substantial role for the nucleolus localization of BLLF2. However, no functional NES was found.

Nuclear export is a strikingly sophisticated and extremely regulated procedure in cells. The members of the importin/exportin family of nucleocytoplasmic transport receptors, such as TAP and CRM1, are essential for the nuclear export of proteins. For example, the nuclear exports of EBV BFLF2 and EB2 (Mta) are accomplished by directly associating with TAP/NXF1 [10, 21]. HSV-1 ICP27, varicella-zoster virus (VZV) IE4 and kaposi’s sarcoma-associated herpesvirus (KSHV) ORF57 can bind to several cellular export factors, including SRp20, ASF/SF2, Aly, 9G8 and TAP, to accelerate the export of viral mRNAs via the
TAP/NXF1 export pathway [9, 38]. Pseudorabies virus (PRV) UL54, influenza A virus (IAV) NS1 and herpesvirus saimiri ORF57 are also demonstrated to achieve mRNA export from the nucleus to cytoplasm by TAP pathway [8, 39–41]. Additionally, HSV-1 UL4, UL47 and VP19C are manifested to transport to the cytoplasm through functional NES mediated by CRM1-dependent pathway [29, 42, 43], which is also exploited by KSHV ORF9, LANA2 and human cytomegalovirus UL94 to fulfill their nuclear exports via a classical NES [44–46] (Fig. 7). Besides, the nuclear export activities of chicken anemia virus VP1, IAV NS2 and human immunodeficiency virus type 1 Rev are also modulated via CRM1-mediated pathway [47–49].

In this study, we found that EBV BLLF2 could shuttle between the cytoplasm and nucleus. Although we did not identify the classical NES in BLLF2, it could not rule out the nuclear export of BLLF2 is mediated through a non-classical pathway or through the interaction with other proteins, or the nucleocytoplasmic shuttling of BLLF2 is mediated by the spatial constituted functional NES. Accordingly, we continued to identify the nuclear export mechanism of BLLF2, and found that its nuclear export neither depends on CRM1 nor TAP. Therefore, the exact nuclear export mechanism of BLLF2 needs to be further explored in the future study.

The classical importin-dependent mechanism for nuclear transport is well known for importin α/β/NLS-cargo complex, of which importin α discriminates the NLS, and importin β executes the association with small regulatory Ran-GTP to ship the complex into the nucleus [5, 22, 50–52]. Moreover, proteins imported into the nucleus also can directly attach to importin β beyond the engagement of importin α-like adaptor [53]. Up to now, some herpesvirus-encoded proteins are reported to be transported into the nucleus by diverse mechanisms. The nuclear translocation of EBV BFLF2 is mediated through Ran-, importin α7-, importin β1- and transportin-1-dependent pathway. EBV EBNA-LP interacts with importin α1 [20], and EBNA1 combines to importin α1 and importin α5 [6], to complete their nuclear traffickings. HSV-1 UL31 is imported into the nucleus through Ran-, importin α1- and transportin-1-mediated pathway [54]. HSV-1 UL2 is also described to be assisted into the nucleus through Ran-, importin α1-, α5-, α7-, β1- and transportin-1 cellular transport receptors [55]. PRV ICP22 is demonstrated to be targeted to the nucleus via Ran-, importin α1-, and α7-mediated pathway [56]. PRV UL54 is proved to accumulate in the nucleus through a classic Ran-, importin β1- and α5-dependent mechanism [8]. In addition, VZV ORF9 and HSV-1 US11 are shown to be transported into the nucleus via Ran- and importin β-dependent pathway [44, 57] (Fig. 7). Here, our data disclosed that the nuclear trafficking of BLLF2 was restrained by the Ran-GTP Q69L mutant, indicating BLLF2 is a Ran-dependent protein. Furthermore, co-transfection of DNAs or inhibitors of importins demonstrated that the nuclear import of BLLF2 is mediated by importin β1.

**Conclusion**

In conclusion, we identified EBV BLLF2 was definitely located in the nucleus and nucleolus, which were achieved by two functional NLSs 16KRQALETVPHPQNRRG31 (NLS1) and 44RRPRPPVAKRRR58 (NLS2) and the nucleolus localization signal 44RRPR47. Additionally, BLLF2 was demonstrated to traffic from the cytoplasm to nucleus via Ran- and importin β1-dependent mechanism, without the participation of
However, the nuclear expert of BLLF2 was mediated neither by CRM1- nor TAP-dependent pathway.

## Methods

### Enzymes and antibodies

All cloning-relevant enzymes were supplied by Thermo Scientific, with the exclusion of KOD-Plus-Neo DNA polymerase and T4 DNA Ligase were afforded by TOYOBO (Japan) and Takara (Beijing, China), respectively. Mouse anti-Flag monoclonal antibody (mAb) and rabbit anti-YFP polyclonal antibody (pAb) were offered by Abmart (Shanghai, China) and RayBiotech, respectively. Mouse nonspecific IgG was purchased from eBioscience (Thermo Fisher Scientific, USA). Alkaline phosphatase (AP)-linked anti-rabbit IgG and anti-mouse IgG were provided by Cell Signaling Technology (MA, USA), and protein A/G PLUS-Agarose was bought from Santa Cruz (Texas, USA).

### Construction of expression plasmids

Plasmid expressing EBV BLLF2 inserted to the C-terminus of EYFP (pEYFP-BLLF2) was constructed in our lab previously [23]. Diverse fragments and mutants of BLLF2 inserted into pEYFP-C1 (Clontech, BD Biosciences) were developed with analogous manner, as described previously [58, 59]. Plasmid expressing BLLF2-Myc (pBLLF2-Myc) and BLLF2-EYFP (pBLLF2-EYFP) were also generated by inserting full-length of BLLF2 fragment into the EcoRI and BamHI digested vectors pMyc-N1 (regenerated from pEYFP-N1) and pEYFP-N1 (Clontech, BD Biosciences), respectively. All constructed clones were validated by sequencing.

Moreover, PRV UL31-EYFP, pEYFP-BFLF2, pUL4-EYFP, pTAP-mCherry, pCRM1-mCherry, pDN kα1-mCherry, pDN kβ1-mCherry, pRan-Q69L-mCherry and pFlag-importin β2 expression plasmids were also constructed in our lab previously [23, 25, 31, 60, 61]. Plasmid pNucleolin-EGFP, a nucleolar marker, was provided by Dr. Johannes A. Schmid (Department of Vascular Biology and Thrombosis Research, University of Vienna Medical School and Competence Center Bio-Molecular Therapeutics). Expression plasmids of Flag-κα2 (importin α1), Flag-κα4 (importin α3), Flag-κα1 (importin α5), Flag-κα6 (importin α7) and pCMV9-3×Flag-importin β1 were provided by Drs. Reinhard Depping (Department of Physiology, University of Lübeck), Yoshihiro Yoneda (Department of Biochemistry, Graduate School of Medicine, Osaka University) and Ben Margolis (Howard Hughes Medical Institute, University of Michigan Medical School), respectively. M9M-RFP and Bimax2-RFP were supplied by Dr. Nobuyuki Nukina (Laboratory for Structural Neuropathology, Brain Science Institute).

### Cell culture, transfection and subcellular localization

HEK393T cells, COS-7 cells and NIH3T3 cells were cultured and transfected by employing polyethylenimine (Sigma, Shanghai, China), as previously described [8, 58]. 24 h post-transfection, cells were washed with PBS and then dyed with 4’,6’-diamidino-2-phenylindole (DAPI) (Thermo Fisher
Scientific, USA) for 5 min, to view the nucleus (blue). Finally, the stained cells were inspected by fluorescence microscope. All subcellular localization experiments were performed at least 3 times, and similar results were obtained.

**Confocal microscopy analysis**

COS-7 cells, cultured on cover-slips in the 24 well plate (Corning, USA), were co-transfected with the indicated expression plasmids for 24 h. Transfected cells were then fixed with 4% paraformaldehyde (Tianjun biotechnology, China) for 30 min. Subsequently, the fixed cells were washed three times with PBS (Boster, Wuhan, China), followed by staining with DAPI for 5 min at 37 °C. Then, the stained cells were placed on the microscope slides (Biosharp, Shanghai, China) for confocal microscope analysis. Samples were assayed through a Leica confocal laser scanning microscope (Leica SP8), by using 63×1.4NA immersion oil lenses, with excitation wavelength at 512 nm for YFP/EGFP, 405 nm for DAPI and 555-580 nm for mCherry/RFP. All confocal microscopy experiments were performed at least 3 times, and similar results were obtained.

**Interspecies heterokaryon assays**

The interspecies heterokaryon assays were performed in accordance with previous studies [8, 10, 25, 62-66]. In short, monkey COS-7 cells were seeded in 6 well plate and transfected with the indicated plasmids. 18 h post-transfection, mouse NIH3T3 cells were added into the COS-7 cells containing cycloheximide (50 μg/mL, inhibiting the synthesis of new proteins) (Sigma, Shanghai, China), which was used to prevent the residual target plasmid entering NIH3T3 cells for expression, to help us analyze whether the target protein can shuttle from COS-7 cells to NIH3T3 cells. In the experiments, cells were treated with or without 20 ng/mL LMB (Sigma, Shanghai, China). 4 h later, polyethylene glycol (Sigma, Shanghai, China) was employed to fuse COS-7 cells with NIH3T3 cells. After hatching for 1 h, cells were stained with DAPI and imaged by fluorescence microscopy. All interspecies heterokaryon assays were performed at least 3 times, and similar results were obtained.

**Co-IP and western blot assays**

Co-immunoprecipitation (Co-IP) and western blot (WB) assays were performed as described previously [8, 44, 54-56, 58, 59, 67, 68]. Briefly, HEK293T cells co-transfected with different expression plasmids combination were collected and lysed with 600 to 800 μL lysis buffer on ice for 30 min, then centrifuged at 12000 g at 4 °C for 15 min. For each Co-IP, supernatants were immunoprecipitated with anti-YFP pAb, anti-Flag mAb or mouse nonspecific IgG at 4 °C for 2 to 4 h, and then incubated overnight with protein A/G beads. The beads were then washed four times with PBS buffer at 2500 g at 4 °C for 5 min. Subsequently, the complex with SDS–PAGE loading buffer was boiled for 10 min and subjected to SDS–PAGE/WB analysis after centrifuging at 12 000 g for 5 min. For WB analysis, the immunoprecipitated proteins were stained with the indicated Abs. Cell lysates were also directly subjected to WB analysis, to verify the expression of specific proteins. All Co-IP and WB assays were performed at least 3 times, and similar results were obtained.
Abbreviations

EBV
Epstein-Barr virus; CRM1: chromosomal region maintenance 1; NLS: nuclear localization signal; NES: nuclear export signal; TAP: transporter associated with antigen processing; NPC: nuclear pore complex; Ran: Ras-related nuclear protein; mRNPs: messenger ribonucleoprotein particles; rRNAs: ribosomal RNAs; snRNAs: small nuclear RNAs; Smad3: mothers against decapentaplegic homolog 3; eIF5A: translation initiation factor 5A; CAS: cellular apoptosis susceptibility protein; EBNA2: EBV nuclear antigen 2; aa: amino acids; DN: dominant negative; LMB: leptomycin B; VZV: varicella-zoster virus; KSHV: Kaposi’s sarcoma-associated herpesvirus; PRV: Pseudorabies virus; IAV: influenza A virus; mAb: monoclonal antibody; pAb: polyclonal antibody; AP: alkaline phosphatase; DAPI: 4',6'-diamidino-2-phenylindole; Co-IP: Co-immunoprecipitation; WB: western blot.

Declarations

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Not applicable.

Authors’ contributions

Conceived and designed the experiments: MLL and MSC. Performed the experiments and analyzed the data: MLL, YJG, YXD, YWL, XWO, TC, JYZ, BLL, LH and SYD. Wrote the paper: MLL and MSC. All authors contributed to and have approved the final manuscript.

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Availability of data and material

The data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication
Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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Figures
Subcellular localization and nucleocytoplasmic shuttling of BLLF2. (A) Potential NESs and NLSs of BLLF2 were predicted by the bioinformatics softwares NetNES 1.1 and PSORT II, respectively. Proteins assigned for trafficking into the nucleus encompass aa targeting sequences termed NLSs [69], and proteins bound for delivery out of the nucleus consist of aa targeting sequences named NESs [70]. The basic aas are arginine (R), histidine (H) and lysine (K). PSORT II adopts the following two standards to dissect target protein: 4 residue pattern (termed ‘pat4’) formed by 4 basic aa (K or R), or formed by three basic aa (K or R) and either H or P; the other (termed ‘pat7’) is a pattern beginning with P and followed within 3 residues by a basic segment including 3 K/R residues out of 4. The identified NLSs 16KRQALETYPHPQNRGR31 (NLS1) and 44RRPPPVAKRRR58 (NLS2) were also indicated. (B) Subcellular distributions of EYFP-BLLF2 and EYFP vector in COS-7 cells. (C) Subcellular distributions of BLLF2-EYFP and BLLF2-Myc in COS-7 cells. (D) Co-expression of EYFP-BLLF2 and pNucleolin-EGFP was observed in COS-7 cells. (E) COS-7 and NIH3T3 cells were stained with DAPI. NIH3T3 cells were identified by their speckled nuclei. (F) Nucleocytoplasmic shuttling of BLLF2 was demonstrated by interspecies heterokaryon assays. EYFP-BFLF2 was used as nucleocytoplasmic shuttling positive control. COS-7 and
NIH3T3 cells were discriminated by nuclear staining with DAPI. NIH3T3 cells were identified by their speckled nuclei (red arrowhead). All scale bars indicate 20 um.

**Figure 2**

Identification of the predicted NES and functional NLS in BLLF2. (A) Schematic diagram of constructs encoding EYFP-tagged wild-type BLLF2 and its deletion mutants aa1-43, 1-47, 1-58, 44-58, 48-58, 48-82, 59-82, 59-148, 83-148, 83-110 and full-length mutant BLLF2(44-47)m. (B) Intracellular localizations of deletion mutants BLLF2(1-58) and BLLF2(59-148) in COS-7 cells. (C) Intracellular localizations of deletion mutants BLLF2(83-143) and BLLF2(83-110) in COS-7 cells. (D) Intracellular localizations of deletion mutants BLLF2(1-47) and BLLF2(1-43) in COS-7 cells. (E) Intracellular localizations of deletion mutants BLLF2(48-82), BLLF2(48-58) and BLLF2(59-82) in COS-7 cells. (F) Intracellular localizations of deletion mutant BLLF2(44-58) and full-length mutant BLLF2(44-47)m in COS-7 cells. All scale bars indicate 20 um.
aa16-31 is another functional NLS of BLLF2. (A) Schematic diagram of constructs encoding EYFP-tagged wild-type BLLF2 and its deletion mutants aa1-20, 21-43, 1-31, 6-31 and 16-31. (B) Intracellular localizations of deletion mutants BLLF2(1-20) and BLLF2(21-43) in COS-7 cells. (C) Intracellular localizations of deletion mutants BLLF2(1-31) in COS-7 cells. (D) Intracellular localizations of deletion mutants BLLF2(6-31) and BLLF2(16-31) in COS-7 cells. All scale bars indicate 20 μm.
Figure 4

Nuclear import mechanism of BLLF2. (A) Individual subcellular localization of Ran-Q69L-mCherry, DN ka1-mCherry, DN kβ1-mCherry, M9M-RFP, Bimax2-RFP or mCherry vector in COS-7 cells. (B) Co-expression of Ran-Q69L-mCherry/EYFP-BLLF2, DN ka1-mCherry/EYFP-BLLF2, DN kβ1-mCherry/EYFP-BLLF2, M9M-RFP/EYFP-BLLF2, Bimax2-RFP/EYFP-BLLF2 or mCherry/EYFP-BLLF2 in COS-7 cells. All scale bars indicate 20 μm.
Figure 5

Nuclear export mechanism of BLLF2. Interspecies heterokaryon assays were performed to analyze nuclear export of BLLF2. Mouse NIH3T3 cells were plated onto the CRM1-dependent positive control UL4 (A), CRM1-independent negative control EYFP vector (B) or pEYFP-BLLF2 (C) transfected COS-7 cells, with or without LMB treatment, as described in Material and methods. Cells were then stained with DAPI and imaged by fluorescence microscopy. NIH3T3 cells were identified by their speckled nuclei (red arrowhead). (D) COS-7 cells were individually transfected with UL4-EYFP or co-transfected with expression plasmids CRM1-mCherry/UL4-EYFP or CRM1-mCherry/EYFP-BLLF2, then examined by confocal microscopy. (E) COS-7 cells were individually transfected with the TAP-dependent positive control EYFP-BFLF2 or co-transfected with expression plasmids TAP-mCherry/EYFP-BFLF2 or TAP-mCherry/EYFP-BLLF2, then examined by confocal microscopy. All scale bars indicate 20 um.
Figure 6

BLLF2 binds to importin β1. (A-G and L) Co-IP analysis of BLLF2 with importin β1 (A and G), importin α5 (ka1) (B), importin α1 (ka2) (C), importin α3 (ka4) (D), importin α7 (ka6) (E), importin β2 (F) or Flag vector (L). HEK293T cells were co-transected with plasmids expressing 3×Flag-importin β1/EYFP-BLLF2 (A and G), Flag-ka1/EYFP-BLLF2 (B), Flag-ka2/EYFP-BLLF2 (C), Flag-ka4/EYFP-BLLF2 (D), Flag-ka6/EYFP-BLLF2 (E), Flag-importin β2/EYFP-BLLF2 (F) or Flag vector/EYFP-BLLF2 (L) for 24 h, cells were subsequently lysed and Co-IPed with anti-Flag mAb (A-F and L) or anti-YFP pAb (G) or mouse control IgG, then WB analysis was carried out with the indicated Abs. (H-I) Co-IP analysis of importin β1 with BFLF2 (H) or PRV UL31 (I). HEK293T cells were co-transected with plasmids expressing 3×Flag-importin β1/EYFP-BFLF2 (H) or 3×Flag-importin β1/PRV UL31-EYFP (I) for 24 h, cells were then lysed and Co-IPed with anti-Flag mAb or mouse control IgG, then WB analysis was carried out with the indicated Abs. (J-K) Co-IP analysis of EYFP vector with Flag vector (J) or importin β1 (K). HEK293T cells were co-transected with plasmids expressing Flag vector/EYFP vector (J) or 3×Flag-importin β1/EYFP vector (K) for 24 h, cells were then lysed and Co-IPed with anti-Flag mAb (J) or anti-YFP (K) or mouse control IgG, then WB analysis was carried out with the indicated Abs.
Figure 7

Schematic diagram of nuclear transport mechanisms of EBV and other herpesviruses-encoded proteins.