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BFRF1 protein is involved in EBV-mediated autophagy manipulation

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Abstract:	Viral egress and autophagy are two mechanisms that seem to be strictly connected in Herpesviruses's biology. Several data suggest that the autophagic machinery facilitates the egress of viral capsids and thus the production of new infectious particles. In the Herpesvirus family, viral nuclear egress is controlled and organized by a well conserved group of proteins named Nuclear Egress Complex (NEC). In the case of EBV, NEC is composed by BFRF1 and BFLF2 proteins, although the alterations of the nuclear host cell architecture are mainly driven by BFRF1, a multifunctional viral protein anchored to the inner nuclear membrane of the host cell. BFRF1 shares a peculiar distribution with several nuclear components and with them it strictly interacts. In this study, we investigated the possible role of BFRF1 in manipulating autophagy, pathway that possibly originates from nucleus, regulating the interplay between autophagy and viral egress.
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Dear Editor Prof. David M. Ojcius

please find enclosed our manuscript by Gonnella et al. entitled:" BFRF1 protein is involved in EBV-mediated autophagy manipulation". In this paper we identify a new role for the EBV early protein BFRF1 in manage autophagic flux during viral egress.

I hope you will find it suitable for publication in Microbes and Infection.

Kind regards

Antonella Farina

BFRF1 protein is involved in EBV-mediated autophagy manipulation.

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Abstract

Viral egress and autophagy are two mechanisms that seem to be strictly connected in Herpesviruses's biology. Several data suggest that the autophagic machinery facilitates the egress of viral capsids and thus the production of new infectious particles. In the Herpesvirus family, viral nuclear egress is controlled and organized by a well conserved group of proteins named Nuclear Egress Complex (NEC). In the case of EBV, NEC is composed by BFRF1 and BFLF2 proteins, although the alterations of the nuclear host cell architecture are mainly driven by BFRF1, a

multifunctional viral protein anchored to the inner nuclear membrane of the host cell. BFRF1 shares a peculiar distribution with several nuclear components and with them it strictly interacts. In this study, we investigated the possible role of BFRF1 in manipulating autophagy, pathway that possibly originates from nucleus, regulating the interplay between autophagy and viral egress.

Keywords: EBV lytic cycle; nuclear egress; autophagy; BFRF1; LC3; lamin B1.

1.Introduction.

Viral maturation occurs in different cellular compartments leading to structural modifications of the host cells. In human Herpesviruses, the induction of the lytic cycle triggers such mechanism starting from the nucleus where the viral DNA is packaged into the capsids that need to cross the nuclear membrane to complete their intracellular maturation. This process, known as "nuclear egress", is mediated by a group of highly conserved viral proteins in all Herpesviruses [1-4]. Epstein-Barr Virus (EBV) is a ubiquitous virus that infects 95% of the world's healthy population, frequently associated to various neoplastic, lymphoproliferative and autoimmune disorders [5, 6]. During lytic replication the assembly of viral infectious progeny provokes remodeling of host cell’s architecture, which is necessary to allow the complete viral

maturation. In the early steps of lytic cycle, EBV encodes two well conserved early proteins, BFRF1 and BFLF2 [7, 8], whose expression and interaction is required for viral nuclear egress. BFRF1, a multifunctional phosphoprotein anchored to the nuclear membrane, tethers BFLF2 on the nuclear rim, and prevents its degradation via proteasomal pathway. Further studies performed in a cell line depleted of BFRF1 gene (F1KO) highlighted its pivotal role in nuclear egress since in its absence viral nucleocapsids are trapped underneath the nuclear membrane [7]. Moreover, BFRF1 binds to lamin B1 and helps to dismantle the nuclear lamina (NL), allowing the passage of the newly assembled nucleocapsids. NL is a fibrillar network located beneath the nuclear envelope that provides the nucleus with mechanical strength, regulates chromatin organization and modulates gene expression and silencing [9]. During EBV replication, BFRF1 alters the distribution of emerin [3, 10], an integral nuclear membrane protein that normally stabilizes the lamina and is involved in induction autophagy [11, 12]. This mechanism is also conserved in KSHV where this role is exerted by ORF67, the positional homologue of BFRF1 [9]. Recent findings reported that BFRF1 has a key role in recruiting the endosomal sorting complex (ESCRT) of the host cell to promote nuclear envelope (NE) alterations inducing the formation of perinuclear vesicles whose nature has not been clarified yet [13, 14].

Recently our group reported that EBV and KSHV are able to hijack the autophagic process to promote their replication [15, 16]. Autophagy is a ubiquitous cellular catabolic process, through which many unwanted cellular components are sequestered in vesicles known as autophagosomes. These vesicles are subsequently merged to lysosomes to be degraded favoring the survival of the cells [17]. In EBV, initial phases of autophagy promote viral lytic cycle while final steps of autophagy are blocked during viral replication. This suggest that the virus manipulates autophagy to facilitate its intracellular transport and to escape degradation. Moreover, it has been shown that ZEBRA, the EBV protein that promotes the initial phases of autophagy, is not able to block final stages of this process [16]. It has also been reported that the block of autophagy may be controlled by proteins of the early phase of viral replication [18] and that EBV uses the autophagic membranes for efficient envelope acquisition during lytic cycle [19].

Since it is known that BFRF1 plays a key role in EBV egress and in altering the distribution of emerin, that may regulate autophagy [12], aim of this study was to evaluate its role in EBV-mediated autophagy manipulation.

2. Materials and Methods

Cells. 293 cell line is an EBV-negative human embryonic epithelial kidney cell line [20], B95-8 is a marmoset, EBV

producing, B-cell line [21]. WT and F1KO are cell lines stably transfected with wt EBV genome and BFRF1 depleted genome respectively [22] [7]. All cell lines were cultured in RPMI 1640, 10% fetal calf serum (FCS) (Aurogene), L-glutamine (2 mM), streptomycin (100 µg/ml), and penicillin (100 U/ml) in 5% CO2 at 37°C. EBV lytic cycle in B95-8 cells have been induced by using TPA 40 ng/ml and Sodium Butyrrate 6 mM.

Cell transfection. Cell line transfection was performed using Xfect transfection kit following the manufacturer's instructions (Clontech #631318). 48 hr post-transfection cells were treated for 3 hours with 20 nM bafilomycin A1 (BAF) (sc-201550; Santa Cruz Biotechnology).

Expression Vectors. The plasmids used are the following: pCMV-F1 [23], pCMVgenZ kindly provided by G. Miller [24] and pEGFP-LC3 [16].

Immunofluorescence. Cells were collected, washed and seeded on multispot slides then fixed and permeabilized as described elsewhere [19]. The following primary antibodies were used: mouse monoclonal anti-BFRF1 (E10, 1:50) and rabbit polyclonal anti-LC3 (Novus biological 1:1000). As secondary antibodies Sheep anti-mouse IgG-Cy3 (SAM-Cy3, Jackson;1:2000), and goat anti-rabbit IgG FITC-conjugated (Jackson, 1:200) were used. Nuclei were stained with DAPI for 1 min RT.

Cell Imaging. Immunofluorescence was analyzed by using an Axio Observer Z1 inverted microscope, equipped with an

ApoTome.2 System (Carl Zeiss Inc., Ober Kochen, Germany). Digital images were acquired with the AxioCam MRm high resolution digital camera (Zeiss) and processed with the AxioVision 4.8.2 software (Zeiss) [25]. ApoTome optical sectioning images of fluorescent cells were recorded under 40×/0.75 objective (Zeiss). Pearson's correlation coefficient was used to quantify the degree of colocalization between fluorescent LC3 and BFRF1 staining in a series of 0.5 nm sequential sections. Pearson's correlation coefficient was calculated using the AxioVision 4.8.2 software (Zeiss), analyzing a minimum of 20 cells randomly taken from each slide from three independent experiments. Images were obtained from the 2D reconstruction of selected serial optical sections.

Western-blot analysis and imuunoprecipitation. Cells were lysed in a RIPA buffer 1x (150mM NaCl, 1% NP-40, 50mM Tris-HCl, pH 8, 0.5% deoxycholic acid, 0.1% SDS, protease and phosphatase inhibitors) on ice for 30 minutes, proteins concentration were measured by using BCA protein assay kit (Sigma 71285-M). Equal amount of each extract was loaded and separated by SDS-PAGE or Nu-PAGE gels (Thermofisher) gels then immunoblotted on nitrocellulose membranes (Protran, Ge Healthcare). The membranes were then blocked in PBS, 0.1% Tween-20, 3% BSA and probed (1hr RT) with specific primary antibodies: mouse monoclonal anti-BFRF1, E10, 1:50; rabbit polyclonal anti-LC3 (Novus Biologicals) 1:1000; rabbit polyclonal anti-BFRF1, R319 1:1000 [26]; rabbit polyclonal anti-Rab7

(Santa Cruz Biotechnology Inc.) 1:300; mouse monoclonal anti-β actin (Santa Cruz Biotechnology Inc.) 1:1000; goat polyclonal anti Lamin B1 (Santa Cruz Biotechnology Inc.) 1:200. After several washes in PBS- 0.1% Tween 20 the membranes were incubated with appropriate polyclonal secondary antibodies HRP-conjugated: polyclonal anti-mouse IgG-HRP (Bethyl, 1:10000), anti-rabbit IgG-HRP (Bethyl, 1:20000) and anti-goat IgG-HRP (SIGMA 1:30.000) for 30 minutes. Finally, the membranes were washed in PBS-0.1% Tween-20 and immunoreactivity was detected using an enhanced chemiluminescence kit (Thermo-Fisher). IP have been performed on insoluble fraction as described elsewhere [8].

3.RESULTS

3.1. BFRF1 promotes the early steps of autophagy.

A previous paper from our group, reported that EBV reactivation from latency blocks the final steps of autophagy (Granato 2014). However, the autophagic block was not mediated by ZEBRA, an EBV immediate-early lytic protein encoded by the BZLF1 gene, whose transfection in EBV negative cell lines Ramos, Akata, and 293 led to a complete autophagic flux. The block occurred only when the complete set of EBV lytic genes was expressed [16]. Moreover, recent findings showed that, in a cell line defective for late antigen expression, an inverse correlation between EBV early antigens expression and autophagosome formation occurred, suggesting that, early after activation of the

lytic cycle, the virus was able to suppress autophagy [18]. To date viral protein/s involved in this mechanism have not been identified. In order to explore this issue, we analyzed the autophagic flux in two cell lines, the WT that harbors a WT EBV genome and the F1KO in which the BFRF1 gene was deleted. In both cell lines, viral replication was induced by BZLF1 (Z) transfection (Fig 1A) and, as a marker of autophagy, we analyzed the level of lipidated form of LC3 (LC3-II) in presence or in absence of Bafilomycin (BAF). As expected, when lytic cycle is activated in the parental cell line (WT+Z), we observed a block of the autophagic flux, as the amount of LC3-II in the presence of BAF didn't change respect to the untreated sample (LC3-II/ β -actin =1,1± BAF). In F1KO cells instead, EBV reactivation (F1KO+Z) led to an accumulation of LC3II following BAF treatment (LC3II/ β -actin= 0.6 vs =0.4), indicating a recovery of the autophagic flux in the absence of BFRF1. These results suggest that the autophagic flux was blocked by BFRF1. We could also observe that, when lytic cycle was activated in the presence of BAF, the accumulation of LC3II was higher in WT cells (WT+Z+BAF) respect to F1KO cells (F1KO+Z+BAF) (LC3II/ β -actin=1.1 vs 0.6) (Fig.1A). Since a higher accumulation of LC3II protein in presence of BAF indicates a higher autophagosome formation, we can infer that, following EBV lytic cycle activation, BFRF1 was able to promote the early steps of autophagy in WT cells. In order to support this hypothesis, we analyzed the autophagic flux during EBV lytic

cycle in F1KO cells in which the missing BFRF1 was restored by transcomplementation (F1KO+Z+F1) (Fig 1B). Following double transfection of BZLF1 and BFRF1 genes, we found that the accumulation of LC3II didn't change upon treatment with BAF (LC3II/ β -actin=0.9 vs 0.8), suggesting that autophagy was again blocked, similarly to what observed in the WT (Fig.1B).

3.2 The downregulation of RAB7 is involved in BFRF1-mediated block of autophagy at the late steps.

Previous data have reported that the block of autophagic flux induced during EBV lytic cycle activation correlated with a reduction of Rab7, a protein responsible for the fusion between autophagic vesicles with lysosomes, in the late steps of autophagy [27]. Therefore, we sought to investigate if the autophagic block induced by BFRF1 could involve the reduction of Rab7 expression. Fig.1C shows an immunoblot performed on WT and F1KO cells induced to lytic replication by BZLF1 transfection where it can be observed that the expression of Rab7 protein increased in the absence of BFRF1. This could explain why in the absence of this protein the autophagic flux was restored.

3.3. BFRF1 shares some colocalization points with LC3 protein on the nuclear membrane and interacts with it.

Recently it has been demonstrated by Deroyer et al. that during ceramide-C16-induced autophagy emerin (EMD), a constitutive

inner nuclear membrane protein, is phosphorylated and binds LC3II leading to an increase of autophagosome formation [12]. Yadav et al. showed that during EBV lytic cycle, BFRF1 expression altered emerin distribution and replaced it, leading to the disassembly of the inner nuclear membrane, a crucial event for EBV nuclear egress [10]. Starting from these considerations, we sought to investigate whether BFRF1 could mimic emerin function in promoting the first steps of autophagy. To this aim, we analyzed BFRF1 distribution respect to LC3 protein by immunofluorescence (IFA). Fig 2B shows a Z-stack image of cells co-transfected with GFP-LC3 and CMV-BFRF1 plasmids. Although most of GFP-LC3 protein was distributed in the cytoplasm, a part of it was localized on the nuclear membrane (green), while BFRF1 was normally distributed on the nuclear rim (red), as expected. Merge panel shows some co-localization points of BFRF1 and LC3 proteins (yellow). Similar results were obtained in B95-8 in which we observed some colocalization points between LC3 and BFRF1, in cells where viral replication was spontaneously activated (Fig 2D Merge). The colocalization of BFRF1 and LC3 on nuclear membrane suggests a possible cooperation between these proteins in autophagic induction during viral replication.

To investigate whether a physical interaction between BFRF1 and LC3 exists, we performed immunoprecipitation (IP) experiments on the insoluble fraction of 293 cells co-transfected with both expression plasmid CMV-BFRF1 and GFP-LC3 (Fig.

3A). IP was performed using the polyclonal ab R319 specific to BFRF1, whereas western blot was carried out using anti LC3 ab and monoclonal antibody E7 (Mo ab E7) specific to BFRF1. As shown in Fig. 3A, the R319 ab was able to co-immunoprecipitate endogenous LC3 as well as BFRF1 protein, indicating that a physical interaction occurs between these proteins in a virus-free cellular context. Based on these results we repeated the IP experiments in a more physiological cellular context where the entire virus genome is present i.e. the WT and in B95-8 cells, in which lytic cycle was activated by BZLF1 transfection or TPA/Na Butyrate treatment, respectively. As shown in Fig. 3B and Fig.3C, also in these cases we were able to observe an interaction between BFRF1 and LC3 (WT+Z IP R319 in Fig 3B and lane B95-8+ in Fig 3C). The R319 antibody, was not able to immunoprecipitate BFRF1 as well as LC3 in a lysate obtained from F1KO cell induced to viral replication, thus confirming the specificity of the interaction between LC3 and BFRF1 (Fig 3B).

3.4. BFRF1 tethers LC3 on lamin B1. Recent findings demonstrated that autophagic machinery plays a role in degradation of nuclear lamina in mammals, a mechanism mediated by a direct interaction between LC3 and lamin B1, a structural component of the Nuclear Lamina (NL) [28, 29]. A previous paper from our group demonstrated that BFRF1 protein interacts and participates in dismantling lamina B1 [8]. On the light of these observations, we asked whether BFRF1 could

interact with both lamin B1 and LC3 to possibly promote nuclear lamina degradation through autophagy, further linking autophagy, viral replication and nuclear egress. To this aim we performed an IP on insoluble fraction of B95-8 cells induced (B+) or non induced (B) to viral replication, using a goat polyclonal ab specific to the lamin B1. Fig 3D shows the western blot analysis carried out following IP in which it is possible to observe that BFRF1 interacted with LC3I and lamin B1. Interestingly, the capacity of lamin B1 to bind LC3-I was reduced in B+ compared to B cells.

Taken together these data suggest that during viral replication LC3I is no longer able to bind directly to lamin B1 but it rather interacts with BFRF1, forming a complex that could promote lamina degradation and facilitate the viral egress.

4. Discussion.

In the present study, we focused our attention on the role of BFRF1 on the interplay between EBV egress and the autophagic pathway. Viruses have evolved sophisticated mechanisms capable of interfering with the autophagic process and are often able to manipulate it in order to promote their replicative cycle [30], immune escape and tumorigenesis [31].

Autophagy and viral egress are strictly related mechanisms during EBV lytic cycle. Previous reports indicated that during viral replication, EBV manipulates autophagy, promoting the early steps while blocking final ones in order to avoid viral

degradation. In that study, we showed that the block of final steps of autophagy was not mediated by the EBV transactivator ZEBRA [16] and other authors reported that the block of autophagy may be controlled by proteins of the early phase of viral replication [18] to which BFRF1 belongs.

BFRF1 is a multifunctional protein with a pivotal role in EBV nuclear egress and it is able to interact with lamin B1 and alter emerin distribution, leading to the dismantling of nuclear lamina [10]. During this process, BFRF1 interacts with the ESCRT (Endosomal Sorting Complex) machinery to remodel the nuclear membrane and induce perinuclear vesicle formation [14]. Furthermore, recent paper described that the ESCRT machinery actively participates in the autophagic process [32].

Thus, the close correlation beetween these events prompted us to investigate whether BFRF1 could be a good candidate in the manipulation of the autophagic pathway during EBV replication. At least three findings support that this was the case. First, the deletion of BFRF1 removed the block of autophagy induced by viral replication and induced an increase of Rab7 expression that, in previous work we have shown to correlate with the inhibition of autophagy during EBV lytic cycle [16]. Interestingly, it has been observed that the reduction of Rab7 prevented viral degradation via autophagy during the replication of KSHV and HBV [33], suggesting that targeting Rab7 could be a common mechanism to inhibit the last autophagic steps by viruses belonging to different families.

Second, although LC3 is generally considered a cytoplasmic protein, it can also localize in the nucleus [28]. Here we observed that, during EBV lytic replication, LC3 partially colocalizes with BFRF1 on nuclear membrane of cells expressing this protein endogenously or ectopically, such interaction is also supported by the results obtained by immunoprecipitation assays. On the bases of these observations, we speculate that BFRF1 tether the autophagic protein LC3 on the nuclear membrane in order to promote the formation of nuclear vesicles in which viral nucleocapsids could be enclosed to facilitate their intracellulat transportation. As far as we know, this is the first report showing a direct interaction between BFRF1 and LC3, thus providing a molecular mechanism through which the virus could manipulate autophagy. Third, lamin B1 has been demonstrated to bind BFRF1 protein thus the trimolecular complex formed by BFRF1, LC3 and lamin B1 could facilitate the dismantling of nuclear envelope favoring the nucleocapsid passage towards the cytoplasm. In addition, in previous studies BFRF1 has been shown to dislocate emerin further facilitating the dismantling of the nuclear envelope and viral egress [34]. This study reinforce the role of BFRF1 in linking the dismantling of nuclear envelope to autophagy, according to the improvement of nuclear envelope dynamics observed in previous study [12, 13, 35].

HSV-1, another member of Herpesvirus family that triggers a particular form of autophagy, uses the nuclear envelope as the

main source of membranes for the formation of autophagic vesicles and such mechanism is promoted by a viral protein that interacts with LC3I [36]. Similarly, here we observed that BFRF1 interacts with LC3I, as shown by IP experiments, allowing us to speculate that this interaction could be crucial to facilitate the viral egress from the nucleus.

In conclusion, this study highlights for first time the key role of early lytic protein BFRF1 in linking autophagy manipulation to EBV nuclear egress. Of note, nuclear membrane alterations are also reported to be strongly involved in tumorigenesis and senescence, indicating that BFRF1, inducing nuclear membrane remodeling, not only facilitates viral replication but also promote the early steps of viral-driven tumorigenesis, in which autophagy and viral replication may also play a role [37] [38] .

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Conflict of interest

The authors have declared that no conflicting interests exist.

Ethical approval

This article does not contain any studies with human participants performed by any of the authors.

Bibliography

[1] Marschall M, Muller YA, Diewald B, Sticht H, Milbradt J. The human cytomegalovirus nuclear egress complex unites multiple functions: Recruitment of effectors, nuclear envelope rearrangement, and docking to nuclear capsids. Rev Med Virol 2017;27.

[2] Newcomb WW, Fontana J, Winkler DC, Cheng N, Heymann JB, Steven AC. The Primary Enveloped Virion of Herpes Simplex Virus 1: Its Role in Nuclear Egress. mBio 2017;8.

[3] Farina A, Santarelli R, Bloise R, Gonnella R, Granato M, Bei R, et al. KSHV ORF67 encoded lytic protein localizes on the nuclear membrane and alters emerin distribution. Virus Res 2013;175:143-50.

[4] Santarelli R, Farina A, Granato M, Gonnella R, Raffa S, Leone L, et al. Identification and characterization of the product encoded by ORF69 of Kaposi's sarcoma-associated herpesvirus. J Virol 2008;82:4562-72.

[5] Severa M, Rizzo F, Srinivasan S, Di Dario M, Giacomini E, Buscarinu MC, et al. A cell type-specific transcriptomic approach to map B cell and monocyte type I interferon-linked pathogenic signatures in Multiple Sclerosis. J Autoimmun 2019;101:1-16.

[6] Farina A, Farina GA. Fresh Insights into Disease Etiology and the Role of Microbial Pathogens. Curr Rheumatol Rep 2016;18:1.

[7] Farina A, Feederle R, Raffa S, Gonnella R, Santarelli R, Frati L, et al. BFRF1 of Epstein-Barr virus is essential for efficient primary viral envelopment and egress. Journal of virology 2005;79:3703-12.

[8] Gonnella R, Farina A, Santarelli R, Raffa S, Feederle R, Bei R, et al. Characterization and intracellular localization of the Epstein-Barr virus protein BFLF2: interactions with BFRF1 and with the nuclear lamina. Journal of virology 2005;79:3713-27.

412 [9] Shimi T, Pfleghaar K, Kojima S, Pack CG, Solovei I, Goldman AE, et al. The A- and B-
413 type nuclear lamin networks: microdomains involved in chromatin organization and
414 transcription. Genes Dev 2008;22:3409-21.
415 [10] Yadav S, Libotte F, Buono E, Valia S, Farina GA, Faggioni A, et al. EBV early lytic
416 protein BFRF1 alters emerin distribution and post-translational modification. Virus Res
417 2017;232:113-22.
418 [11] Koch AJ, Holaska JM. Emerin in health and disease. Semin Cell Dev Biol 2014;29:95-
419 106.
420 [12] Deroyer C, Renert AF, Merville MP, Fillet M. New role for EMD (emerin), a key inner
421 nuclear membrane protein, as an enhancer of autophagosome formation in the C16-
422 ceramide autophagy pathway. Autophagy 2014;10:1229-40.
423 [13] Lee CP, Liu GT, Kung HN, Liu PT, Liao YT, Chow LP, et al. The Ubiquitin Ligase Itch
424 and Ubiquitination Regulate BFRF1-Mediated Nuclear Envelope Modification for Epstein-
425 Barr Virus Maturation. Journal of virology 2016;90:8994-9007.
426 [14] Lee CP, Liu PT, Kung HN, Su MT, Chua HH, Chang YH, et al. The ESCRT machinery
427 is recruited by the viral BFRF1 protein to the nucleus-associated membrane for the
428 maturation of Epstein-Barr Virus. PLoS Pathog 2012;8:e1002904.
429 [15] Granato M, Santarelli R, Filardi M, Gonnella R, Farina A, Torrisi MR, et al. The
430 activation of KSHV lytic cycle blocks autophagy in PEL cells. Autophagy 2015;11:1978-86.
431 [16] Granato M, Santarelli R, Farina A, Gonnella R, Lotti LV, Faggioni A, et al. Epstein-barr
432 virus blocks the autophagic flux and appropriates the autophagic machinery to enhance
433 viral replication. Journal of virology 2014;88:12715-26.
434 [17] Yang Y, Klionsky DJ. Autophagy and disease: unanswered questions. Cell Death
435 Differ 2020.

436 [18] De Leo A, Colavita F, Ciccosanti F, Fimia GM, Lieberman PM, Mattia E. Inhibition of
437 autophagy in EBV-positive Burkitt's lymphoma cells enhances EBV lytic genes expression
438 and replication. Cell Death Dis 2015;6:e1876.
439 [19] Nowag H, Guhl B, Thriene K, Romao S, Ziegler U, Dengjel J, et al. Macroautophagy
440 Proteins Assist Epstein Barr Virus Production and Get Incorporated Into the Virus
441 Particles. EBioMedicine 2014;1:116-25.
442 [20] Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line
443 transformed by DNA from human adenovirus type 5. J Gen Virol 1977;36:59-74.
444 [21] Gussander E, Adams A. Intracellular state of Epstein-Barr virus DNA in producer cell
445 lines. J Gen Virol 1979;45:331-40.
446 [22] Feederle R, Bartlett EJ, Delecluse HJ. Epstein-Barr virus genetics: talking about the
447 BAC generation. Herpesviridae 2010;1:6.
448 [23] Farina A, Cardinali G, Santarelli R, Gonnella R, Webster-Cyriaque J, Bei R, et al.
449 Intracellular localization of the Epstein-Barr virus BFRF1 gene product in lymphoid cell
450 lines and oral hairy leukoplakia lesions. J Med Virol 2004;72:102-11.
451 [24] Grogan E, Jenson H, Countryman J, Heston L, Gradoville L, Miller G. Transfection of
452 a rearranged viral DNA fragment, WZhet, stably converts latent Epstein-Barr viral infection
453 to productive infection in lymphoid cells. Proc Natl Acad Sci U S A 1987;84:1332-6.
454 [25] Montanari E, Oates A, Di Meo C, Meade J, Cerrone R, Francioso A, et al. Hyaluronan-
455 Based Nanohydrogels for Targeting Intracellular S. Aureus in Human Keratinocytes. Adv
456 Healthc Mater 2018;7:e1701483.
457 [26] Farina A, Santarelli R, Gonnella R, Bei R, Muraro R, Cardinali G, et al. The BFRF1
458 gene of Epstein-Barr virus encodes a novel protein. J Virol 2000;74:3235-44.
459 [27] Kuchitsu Y, Fukuda M. Revisiting Rab7 Functions in Mammalian Autophagy: Rab7
460 Knockout Studies. Cells 2018;7.

461 [28] Dou Z, Xu C, Donahue G, Shimi T, Pan JA, Zhu J, et al. Autophagy mediates
462 degradation of nuclear lamina. Nature 2015;527:105-9.
463 [29] Park YE, Hayashi YK, Bonne G, Arimura T, Noguchi S, Nonaka I, et al. Autophagic
464 degradation of nuclear components in mammalian cells. Autophagy 2009;5:795-804.
465 [30] Choi Y, Bowman JW, Jung JU. Autophagy during viral infection - a double-edged
466 sword. Nat Rev Microbiol 2018;16:341-54.
467 [31] Cirone M. EBV and KSHV Infection Dysregulates Autophagy to Optimize Viral
468 Replication, Prevent Immune Recognition and Promote Tumorigenesis. Viruses 2018;10.
469 [32] Lefebvre C, Legouis R, Culetto E. ESCRT and autophagies: Endosomal functions and
470 beyond. Seminars in cell & developmental biology 2018;74:21-8.
471 [33] Zhou T, Jin M, Ding Y, Zhang Y, Sun Y, Huang S, et al. Hepatitis B virus dampens
472 autophagy maturation via negative regulation of Rab7 expression. Biosci Trends
473 2016;10:244-50.
474 [34] Sakaki M, Koike H, Takahashi N, Sasagawa N, Tomioka S, Arahata K, et al.
475 Interaction between emerin and nuclear lamins. J Biochem 2001;129:321-7.
476 [35] Liu GT, Kung HN, Chen CK, Huang C, Wang YL, Yu CP, et al. Improving nuclear
477 envelope dynamics by EBV BFRF1 facilitates intranuclear component clearance through
478 autophagy. FASEB journal : official publication of the Federation of American Societies for
479 Experimental Biology 2018;32:3968-83.
480 [36] Radtke K, English L, Rondeau C, Leib D, Lippe R, Desjardins M. Inhibition of the host
481 translation shutoff response by herpes simplex virus 1 triggers nuclear envelope-derived
482 autophagy. J Virol 2013;87:3990-7.
483 [37] Hong GK, Gulley ML, Feng WH, Delecluse HJ, Holley-Guthrie E, Kenney SC. Epstein-
484 Barr virus lytic infection contributes to lymphoproliferative disease in a SCID mouse model.
485 J Virol 2005;79:13993-4003.

[38] Cirone M, Gilardini Montani MS, Granato M, Garufi A, Faggioni A, D'Orazi G. Autophagy manipulation as a strategy for efficient anticancer therapies: possible consequences. J Exp Clin Cancer Res 2019;38:262.

Legend to Figures

Fig.1. BFRF1 promotes the early steps of autophagy and blocks final one in WT cell line. **(A)** WT cell line, harboring a WT-EBV genome, and F1KO cell line, carrying a BFRF1 deleted EBV genome, were induced to viral replication by BZLF1(Z) transfection. The expression level of LC3II was detected in the presence or in absence of Bafilomycin (BAF) by western blot analysis and β-actin was used as loading control. **(B)** Trans complementation of BFRF1 gene restores autophagic block in F1KO cells induced to replication by Z transfection. The histograms represent the mean plus S.D. of the densitometric analysis of the ratio of LC3II/β-actin. One representative experiment out of three is shown **(C)** WT and F1KO were transfected with BZLF1 (Z) and, 48 hours later, the expression of Rab7 was monitored by western blot analysis. One representative experiment out of three is shown. Histograms represent the mean plus S.D. of the densitometric analysis of the ratio of Rab7/ β-actin.

Fig.2. BFRF1 shares some colocalization points with LC3 on the nuclear membrane. (A) Immunofluorescence analysis

(IFA) performed on 293 cells (A) and (B) 293 cells co-transfected with GFP-LC3 (green) and BFRF1 (red); colocalization is shown in merged panel (yellow). **(C-D)** IFA performed on spontaneously replicating B95-8 cells: LC3 (green) and BFRF1 (red). Colocalization points are shown in merged panel (yellow). Images were obtained with an ApoTome system (Zeiss, Oberkochen, Germany) and were generated by stacking multiple section scans, one out of 10 optical sections is shown. Image analysis of optical sections was performed by Axiovision software (Zeiss, Oberkochen, Germany). ApoTome micrographs (scale bar: 10 μm). Pearson's correlation coefficient was calculated using the AxioVision 4.8.2 software (Zeiss) and expressed as the mean value ± standard deviation. Results were obtained from three independent experiments, each derived from eight images. Statistical significance was determined with Graph Prism 5 Software. p values < 0.05 were considered significant. Asterisk denotes statistically significant differences (***p < 0.05).

Fig.3. BFRF1 interacts with LC3 and lamin B1. (A) Protein extracts from 293 cells contrasfected with BFRF1 and GFP-LC3 were immunoprecipitated by using anti-BFRF1 polyclonal R319 antibody (IP). Immunoprecipitated proteins were detected by western blot probed with anti LC3 and anti BFRF1 antibodies. The immunoblot shows the endogenous LC3 (14-16 kDa) and BFRF1 (37,5-38 kDa) proteins. As negative control we used IP

w/o antibodies (no ab) and crude lysate (lys). **(B)** Protein extracts from F1KO and WT cells induced to lytic cycle by ZEBRA transfection, were immunoprecipitated by using anti BFRF1 polyclonal R319 ab (IP). Immunoprecipitated proteins were detected by western blot analysis with anti LC3 and anti BFRF1 antibodies. We used IP w/o antibodies (no ab) and crude lysate (lys) of WT cells as negative and positive controls respectively. **(C)** Protein extracts from B95-8 cells induced to lytic cycle (B95-8+) were immunoprecipitated by using anti BFRF1 polyclonal antibody (IP). Immunoprecipitated proteins were detected in western blot by using anti BFRF1 and anti LC3 antibodies, IP w/o antibodies (no ab) and crude lysate (lys) were used as negative and positive controls respectively. **(D)** Protein extracts from B95-8 cells (B) and B95-8 cells induced to lytic cycle (B+) were immunoprecipitated by using anti lamin B1 antibody (LB1). Immunoprecipitated proteins were detected in western blot by using anti BFRF1, anti LC3 and anti LB1 antibodies. IP without antibodies and Flow Throught fraction (FT) were used as controls. All the IP performed have been repeated at least three time.

Figure 1

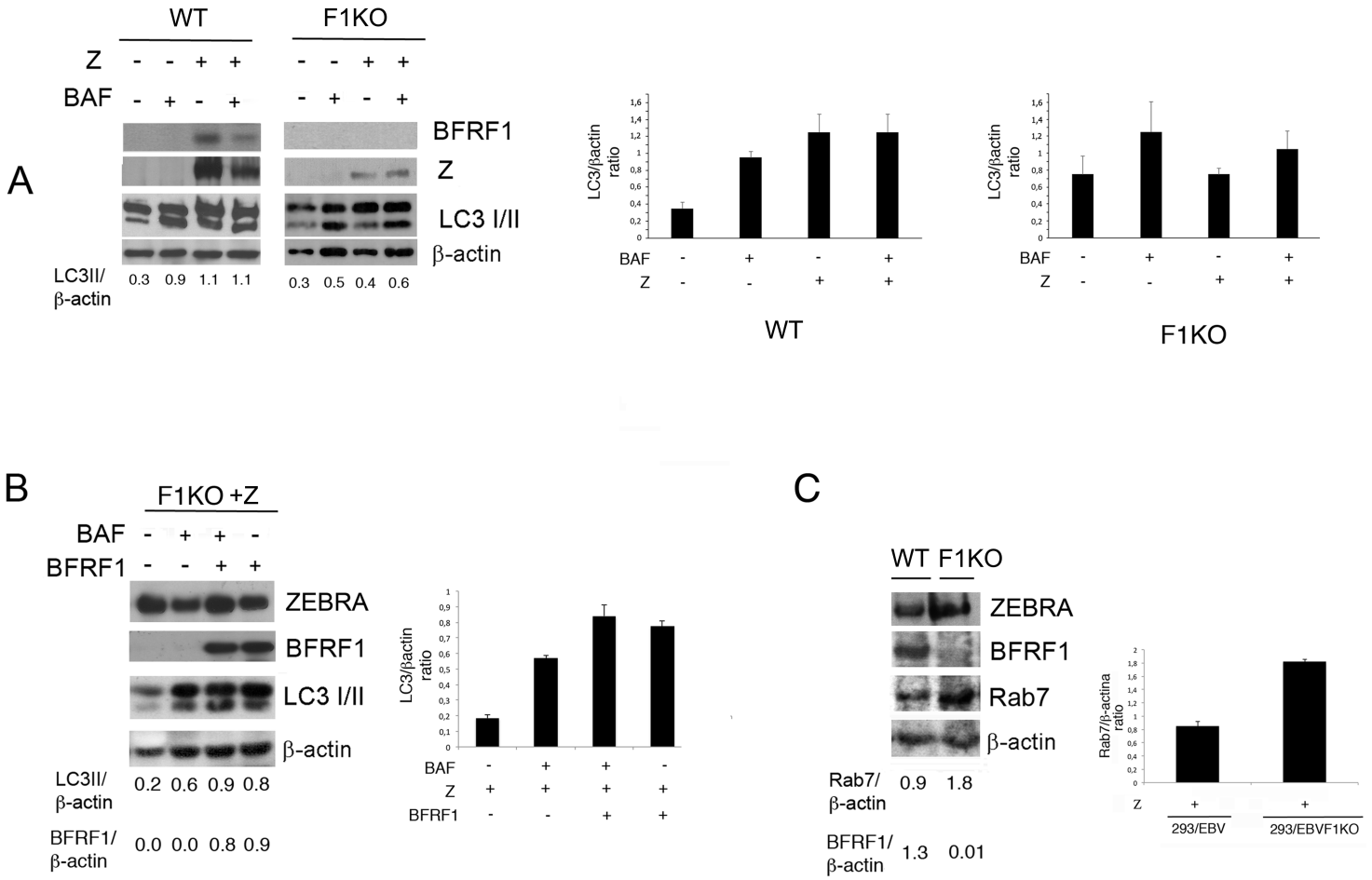

Fig.1

Figure 2

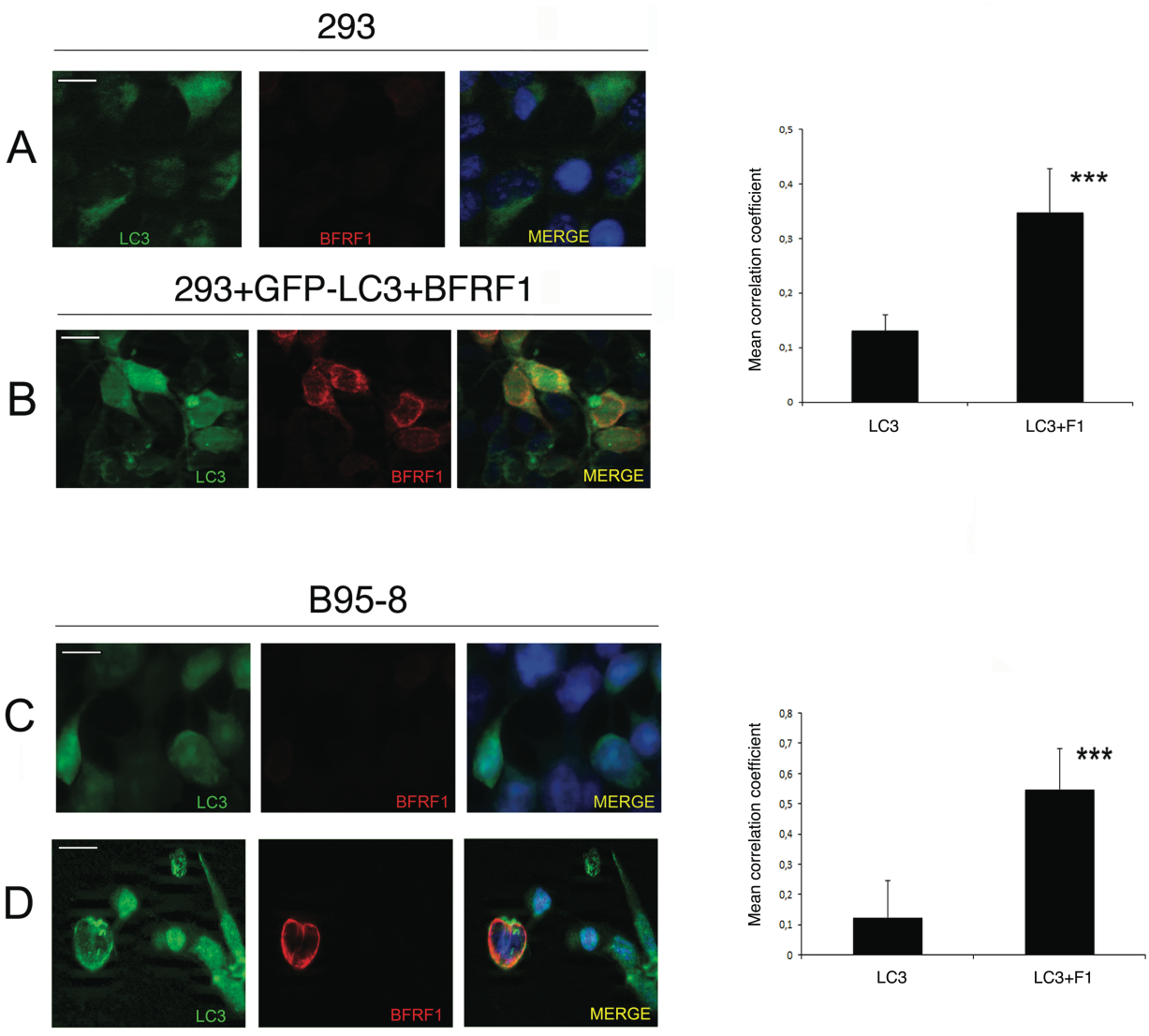

Fig.2

Figure 3

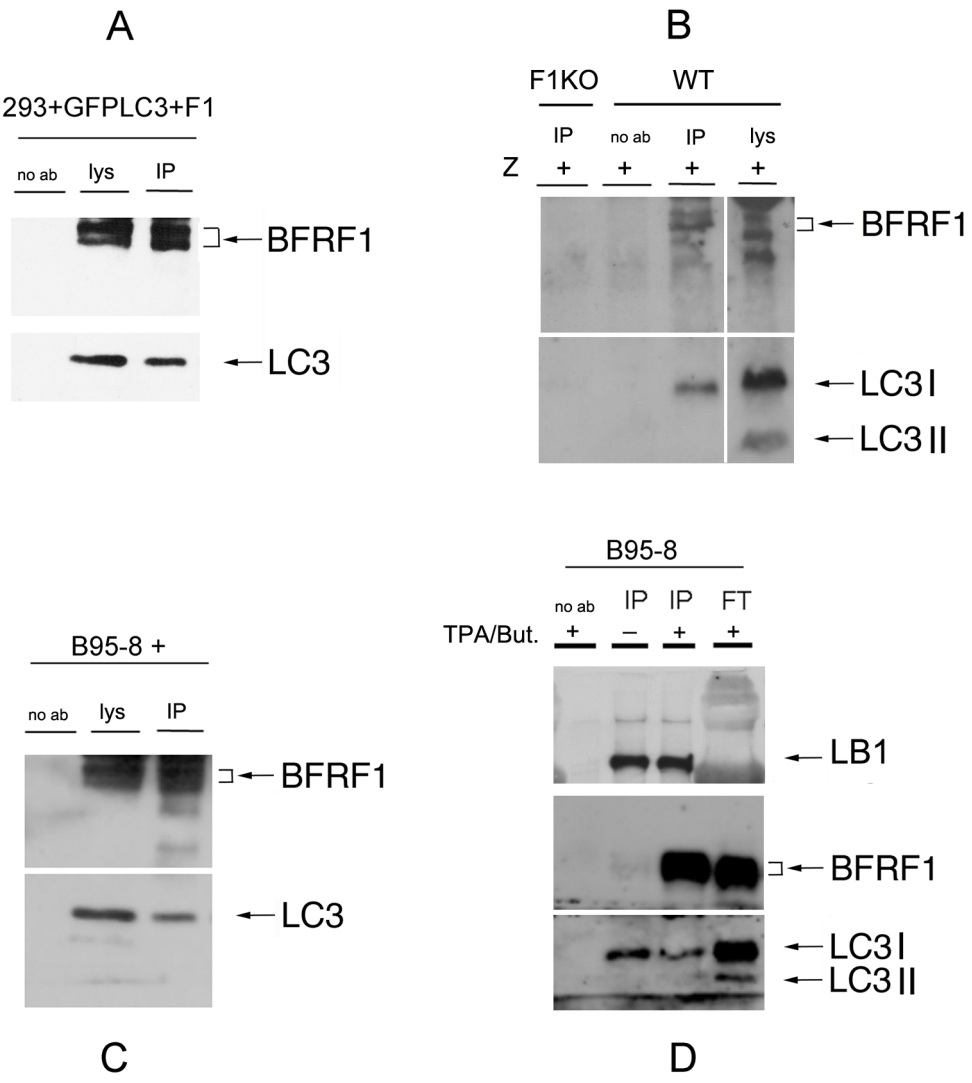

Fig.3