Essential role of hyperacetylated microtubules in innate immunity escape orchestrated by the EBV-encoded BHRF1 protein

Damien Glon¹, Géraldine Vilmen^{1, 2†}, Daniel Perdiz^{3†}, Eva Hernandez¹, Guillaume Beauclair¹, Vincent Maréchal², Christian Poüs^{3, 4}, Marion Lussignol^{1#} and Audrey Esclatine^{1#*}

1. Université Paris-Saclay, CEA, CNRS, Institute for Integrative Biology of the Cell (I2BC), 91198, Gif-sur-Yvette, France

2. CRSA, Centre de Recherche Saint-Antoine, UMRS 938, INSERM, Sorbonne Université, 75012 Paris, France

3. INSERM UMR-S 1193, Université Paris-Saclay, 92296 Châtenay-Malabry, France

4. Biochimie-Hormonologie, APHP, Hôpitaux Universitaires Paris-Saclay, Site Antoine Bécère, 92141 Clamart, France

[†] These authors contributed equally to this work.

[#] These authors share senior authorship.

* For correspondence: audrey.esclatine@universite-paris-saclay.fr (AE)

Competing interests: The authors declare that no competing interests exist.

Abstract

Innate immunity constitutes the first line of defense against viruses, in which mitochondria play an important role in the induction of the interferon (IFN) response. BHRF1, a multifunctional viral protein expressed during Epstein-Barr virus reactivation, modulates mitochondrial dynamics and disrupts the IFN signaling pathway. Mitochondria are mobile organelles that move through the cytoplasm thanks to the cytoskeleton and in particular the microtubule (MT) network. MTs undergo various post-translational modifications, among them tubulin acetylation. In this study, we demonstrated that BHRF1 induces MT hyperacetylation to escape innate immunity. Indeed, expression of BHRF1 induces the aggregation of shortened mitochondria next to the nucleus. This mito-aggresome is organized around the centrosome and its formation is MT-dependent. We also observed that the BHRF1-induced hyperacetylation of MTs involves the α -tubulin acetyltransferase ATAT1. Thanks to a non-acetyltable α -tubulin mutant, we demonstrated that this hyperacetylation is necessary for the mito-aggresome formation. We investigated the mechanism leading to the clustering of mitochondria, and we identified dyneins as motors that are required for the mitochondrial aggregation. Finally, we demonstrated that BHRF1 needs MT hyperacetylation to block the induction of the IFN response. Indeed, in the absence of MT hyperacetylation, BHRF1 is unable to initiate the sequestration of mitochondria into autophagosomes, and mitophagy, which is essential to inhibiting the signaling pathway. Therefore, our results reveal the role of the MT network, and its acetylation level, in the induction of a pro-viral mitophagy.

Introduction

The innate immunity system provides the first line of defense against different invading pathogens. This process is based on the sensing of motifs from the foreign organism, called the pathogen-associated molecular pattern (PAMP), by different host pattern recognition receptors (PRR). In the case of a viral infection, their recognitions notably lead to the induction of the interferon (IFN) response, which leads to the expression of interferon-stimulated genes and the synthesis of cytokines known for their antiviral properties (Lee and Ashkar, 2018).

Mitochondria carry out a crucial role in many cellular processes ranging from energy production to programmed cell death, from calcium homeostasis to cell immunity. They constitute a platform for signaling pathways involved in innate immunity thanks to the MAVS (mitochondrial antiviral signaling protein) receptor, predominantly localized at the mitochondrial outer membrane surface (Arnoult *et al.*, 2011; West *et al.*, 2011). The functions of mitochondria depend on their morphology, which is in turn dependent upon mitochondrial dynamics (including fission, fusion, and motility). Indeed, mitochondria are dynamic organelles that continually switch between cycles of fusion and fission to regulate the network structure (Giacomello *et al.*, 2020). Fission occurs at mitochondria-endoplasmic reticulum (ER) contact sites, which facilitate the recruitment of Drp1 (dynamin related protein 1), which multimerizes and in turn triggers the severing of mitochondria (Pagliuso *et al.*, 2018). Fusion is regulated by both MFN1/2 (mitofusins 1 and 2) and OPA1 (optic atrophy type 1) located at the outer and inner mitochondrial membrane, respectively. Moreover, mitochondria are motile because they are actively recruited to specific cellular locations to respond to their functions. In eukaryotic cells, the cytoskeleton and notably microtubules (MTs) play a critical role in the distribution of mitochondria throughout the cytoplasm by facilitating their transport to areas with high metabolic demands (Frederick and Shaw, 2007; Kruppa and Buss, 2021).

The MT cytoskeleton is a highly dynamic polymer of α and β -tubulin heterodimers, which is involved in a variety of cellular functions, such as supporting cell structures, maintaining cell polarity, but also allowing the transport of organelles. Moreover, it has been shown that the MT network of viral infected cells could be used for intracellular transport of viral particle/genomic material to the sites of replication or assembly (Zheng *et al.*, 2017). A broad range of MT-associated proteins (MAPs) contribute to regulate MT dynamic behavior. MAPs include kinesins and dyneins, which both allow the positioning of organelles and the long-distance transport of vesicles along MT tracks. Kinesins generally transport their cargos toward the plus-end of MTs at the cell periphery. Conversely, dyneins transport their cargos to the minus-end of MTs in the cell center (Hancock, 2014). The relationship between MTs and mitochondrial transport is not completely understood. Mitochondrial movement involves

mitochondrial adaptors such as small mitochondrial Rho GTPases Miro1/2 and TRAK1/2 (López-Doménech *et al.*, 2018). Indeed, Miro proteins can engage both kinesin and dynein to mediate the bidirectional movement of mitochondria along MT tracts (Fransson *et al.*, 2006; Frederick and Shaw, 2007; Tang, 2018).

MT functions are regulated by numerous post-translational modifications such as acetylation, detyrosination, phosphorylation, methylation or polyglutamylolation (Janke and Magiera, 2020). Among them the acetylation of α -tubulin, which mainly occurs on the Lysine in position 40 (K40), has emerged as an important regulator of numerous cell functions (Perdiz *et al.*, 2011). For example, α -tubulin acetylation, which is often linked to MT stabilization, may modulate the binding of MAPs (Dompierre *et al.*, 2007; Mackeh *et al.*, 2014; Reed *et al.*, 2006) and therefore may modulate organelle movement. Indeed, stable MTs are particularly involved in mitochondrial transport (Friedman *et al.*, 2010), and it has been reported that acetylation of α -tubulin mediates dynein-dependent transport of mitochondria along MTs (Misawa *et al.*, 2013).

In this work, we aimed at studying the role of the MT network in the innate immunity escape induced by the Epstein-Barr virus (EBV). BHRF1 is a viral Bcl-2 homolog (Khanim *et al.*, 1997), and our group recently unravelled its novel functions in innate immunity, related to its mitochondrial localization (Vilmen *et al.*, 2020). We demonstrated that BHRF1 disturbs mitochondrial dynamics and subsequently stimulates autophagy. More precisely, BHRF1 induces mitophagy, a cellular process that can specifically sequester and degrade mitochondria by autophagy, and we have shown that BHRF1-induced mitophagy participates in the inhibition of type I IFN response by EBV (Vilmen *et al.*, 2020). Here, we described the importance of the MT hyperacetylation on the mitochondrial clustering and, more unexpectedly, on the capability of BHRF1 to counteract the IFN induction.

Results

BHRF1-induced mito-aggresomes are organized around the centrosomes

The EBV-encoded BHRF1 localizes to mitochondria, and its expression in HeLa cells dramatically alters mitochondrial dynamics and distribution, (Vilmen *et al.*, 2020). By confocal microscopy, we observed abnormal mitochondrial aggregates close to the nucleus in BHRF1-expressing cells, whereas in control cells (empty vector [EV] transfection), mitochondria remained distributed throughout the cytoplasm (**Figure 1A**). This mitochondrial phenotype, known as mito-aggresome, is in accordance with our previous observations. We arbitrarily consider that, when the compaction index (CI) of mitochondria is above 0.4, the cell presents a mito-aggresome, whereas when the CI value is below 0.4, mitochondria are distributed homogeneously throughout the cell cytoplasm (Narendra *et al.*, 2010; Vilmen *et al.*, 2020). The expression of BHRF1 significantly increased the CI values, which corresponds to the formation of a mito-aggresome in almost 80% of the cells (**Figure 1B**). Since BHRF1 is associated with mitochondrial fragmentation (**Figure 1A - insets**), we precisely evaluated mitochondrial shape by calculation of the aspect ratio (AR) and the form factor (FF), two parameters which reflect the mitochondrial length and the branching of mitochondria, respectively. As calculated in **Figure 1C**, BHRF1 expression significantly reduced both AR and FF parameters, confirming an induction of mitochondrial fission.

As mitochondria localize near the nucleus, the involvement of the MT network in this mitochondrial redistribution was investigated. In mammalian cells, mitochondria move along cytoskeletal tracks to sites of high-energy demand in an MT-dependent manner (Detmer and Chan, 2007). At interphase, the MT network radiates from the centrosome, the main MT organizing center for directing the polarity and the orientation of MTs. By co-staining BHRF1-expressing cells for mitochondria and pericentrin, a classical centrosomal marker, we observed that mitochondria are concentrated around the centrosome to form a mito-aggresome (**Figure 1D**). Moreover, a 3D reconstruction confirmed the clustering of BHRF1 in the vicinity of the nucleus around the centrosome (**Supplementary file 1**).

The MT network is required for BHRF1-induced mito-aggresome formation

To test whether MTs play a role in the mitochondrial redistribution following BHRF1 expression, the whole MT network was disassembled using a treatment mixing nocodazole, a classical MT-destabilizing drug, and exposure to cold (Geeraert *et al.*, 2010). As shown in **Figure 1E - left panel** this treatment disassembled MTs (fuzzy staining of α -tubulin). The removal of nocodazole followed by 1 hour in a warm complete medium at 37°C restored the MT network (**Figure 1E - right panel**). In cells without BHRF1 expression, mitochondria were homogeneously distributed in the cytoplasm, and the disorganization of the MT network had no impact on their distribution. Conversely, mitochondrial

aggregation in BHRF1-expressing cells was abolished when MTs were depolymerized (*Figure 1E - left panel*) and mitochondria were then distributed throughout the cell (CI values mainly below 0.4). This phenomenon was reversible since the mitochondria rapidly re-aggregated to different extents within 1 hour after reassembly of the MT network (most of the CI values above 0.4) (*Figure 1E - right panel*). In contrast, mitochondria in non-transfected neighboring cells remained scattered within the cytoplasm after nocodazole removal. The quantification of mito-aggresomes in BHRF1-positive cells showed that whereas less than 20% of the cells presented mitochondrial aggregation after depolymerization treatment (*Figure 1F*), this proportion went back to more than 50% after repolymerization of the MTs (compare to 80% without treatment). We concluded that the MT network is required for the mito-aggresome formation.

As the actin network is also known to be involved in mitochondrial transport and fission (De Vos *et al.*, 2005; López-Doménech *et al.*, 2018), we also checked if F-actin disorganization affects the mitochondrial phenotype induced by BHRF1. In order to do so, we treated HeLa cells with cytochalasin B to disrupt F-actin but we did not observe any changes in the mitochondrial phenotype in BHRF1-positive cells (*Supplementary file 2A*). BHRF1 still induced the mitochondrial aggregation and fission at the same level as in the control (*Supplementary file 2B and C*). Therefore, the actin network does not seem to be involved in the mitochondrial phenotype.

BHRF1 induces MT hyperacetylation, which is required for the mito-aggresome formation

We next explored whether BHRF1 could modify the MT network by assessing its acetylation level. Upon BHRF1 expression, whereas α -tubulin staining was unchanged, acetyl- α -tubulin was redistributed and colocalized with BHRF1 (*Figure 2A*). Moreover, we observed a brighter signal of acetyl- α -tubulin, suggesting an increase in α -tubulin acetylation. This was confirmed by western-blot analysis, showing an increase of 60% under BHRF1 expression (*Figure 2B*).

To determine whether this hyperacetylation could be involved in BHRF1-induced mitochondrial alterations, we expressed a non-acetylatable α -tubulin mutant (mCherry- α -tubulin K40A) which prevents MT hyperacetylation (Dompierre *et al.*, 2007). It has previously been reported that this non-acetylatable α -tubulin mutant does not alter the MT network and is present in the entire MT network (Geeraert *et al.*, 2010). Notably, when α -tubulin K40A is expressed, endogenous α -tubulin still remains acetylatable. However, the MT network's ability to be hyperacetylated is prevented, since α -tubulin acetylation only occurs on protofilaments and the MT network is totally decorated with this non-acetylatable α -tubulin. We thus verified by immunoblot analysis that BHRF1 was unable to induce hyperacetylation of α -tubulin when the mutant α -tubulin K40A was expressed (*Figure 2C*). Then, the impact of α -tubulin K40A on the clustering of mitochondria was analyzed (*Figures 2D and E*). In cells

co-expressing α -tubulin K40A and BHRF1, CI mean value was below 0.4 and mitochondria were homogeneously distributed throughout the cytoplasm, similarly to control cells. Thus, we concluded that BHRF1-induced mito-aggresome formation depends on MT hyperacetylation.

Interestingly, we observed that without α -tubulin hyperacetylation, BHRF1 was still able to promote mitochondrial fragmentation (**Figure 2F**). Confocal images (**Figure 2D, insets**) clearly showed that upon BHRF1 expression (for both WT and K40A conditions) mitochondria were drastically smaller. Therefore, hyperacetylation of MTs is essential for BHRF1-induced mito-aggresome formation but not for mitochondrial fission.

MT hyperacetylation is necessary to the inhibition of type I IFN response by BHRF1

Mitochondria appear to be central organelles for the induction of antiviral innate immunity. RIG-I (retinoic acid-inducible gene) and MDA5 (melanoma differentiation-associated protein 5) are two cytoplasmic PRRs that detect viral genome. Upon activation, these receptors are translocated to the mitochondria to interact with the mitochondrial-resident protein MAVS that recruits and activates TBK1 (TANK-binding kinase 1). This kinase is required for the phosphorylation of the transcription factors IRF3 and IRF7 (interferon regulatory factors 3 and 7), leading to their dimerization, nuclear translocation and the subsequent activation of type I IFN promoter (Arnoult *et al.*, 2011; West *et al.*, 2011). Our group and others previously demonstrated that impairing the mitochondrial morphodynamics leads to the inhibition of IFN response (Castanier *et al.*, 2010; Chatel-Chaix *et al.*, 2016; Vilmen *et al.*, 2020; Yoshizumi *et al.*, 2014). Considering the importance of tubulin hyperacetylation for the formation of mito-aggresomes, we sought to know whether MT hyperacetylation was required for the inhibition of type I IFN response by BHRF1. We performed an IFN- β promoter-driven luciferase reporter assay in BHRF1-expressing HEK293T cells (**Figure 3A**). To activate the IFN-signaling pathway, we expressed an active form of RIG-I, designated Δ RIG-I (2xCARD) (Yoneyama *et al.*, 2004), which constitutively activates MAVS-dependent pathway through CARD domains interaction. As previously observed, BHRF1 dramatically blocked the IFN- β promoter activation, however, the expression of the α -tubulin K40A significantly prevented this inhibition (**Figure 3A**). To confirm our results, we examined the cellular localization of IRF3 by immunofluorescence. In basal conditions, IRF3 staining is diffuse throughout the cytoplasm. When Δ RIG-I (2xCARD) is expressed, IRF3 translocates from the cytoplasm into the nucleus but expression of BHRF1 blocked this translocation (**Figure 3B**). However, when MT hyperacetylation was prevented, BHRF1 was not able to block IRF3 nuclear translocation (**Figure 3B**). Quantification of this assay showed that expression of the non-acetylatable form of α -tubulin totally restored IRF3 nuclear translocation upon BHRF1 expression (**Figure 3C**). Therefore, our results demonstrated that MT hyperacetylation is required for BHRF1 to inhibit type I IFN response.

BHRF1 requires acetyltransferase ATAT1 to modify MTs and form mito-aggresomes.

Thereafter, we wanted to characterize the mechanism of MT hyperacetylation induction by BHRF1. Tubulin acetylation results from the balance between the activities of tubulin acetyltransferases and deacetylases. Therefore, a hyperacetylation of the MTs can be the consequence of either the inhibition of deacetylases or the activation of an acetyltransferase. First of all, we wanted to investigate a potential inhibitory role of BHRF1 on the two main α -tubulin deacetylases, HDAC6 (histone deacetylase 6) and SIRT2 (sirtuin type 2) (Hubbert *et al.*, 2002; North *et al.*, 2003). Indeed, it has been observed that knockdown of SIRT2 results in an aberrant mitochondrial distribution in the perinuclear region (Misawa *et al.*, 2013), and another study showed that the depletion of SIRT2 results in nuclear envelope shape defects (Kaufmann *et al.*, 2016). These observations are clearly similar to the effect of BHRF1 on mitochondria, as well as its distribution around the nuclear envelope and the modification of the nucleus shape (**Figure 1A**). Thus, we performed an *in vitro* tubulin deacetylation assay (Mackeh *et al.*, 2014). This test consists of mixing the highly acetylated porcine brain tubulin with lysates of BHRF1-expressing cells. Lysate of control EV-transfected cells reduces the acetylation of tubulin by approximately 50% compared to condition without cell lysate (**Figure 4A**). The same deacetylase activity is observed with BHRF1-positive lysates, indicating that BHRF1 does not inhibit the deacetylases.

We subsequently investigated the role of ATAT1 (alpha-tubulin N-acetyltransferase 1) (Shida *et al.*, 2010). We knocked down ATAT1 expression by a siRNA approach and observed no more detectable acetylation activity under BHRF1 expression (**Figure 4B**), indicating involvement of ATAT1 in BHRF1-induced hyperacetylation. Then, we checked the mitochondrial network and observed that the knockdown of ATAT1 prevents the mito-aggresome formation but not mitochondrial fission (**Figure 4C-E**). These results led us to conclude that BHRF1 activates the acetyltransferase ATAT1, leading to MT hyperacetylation and subsequent mito-aggresome formation.

Mito-aggresome formation requires retrograde transport of mitochondria via dyneins

As observed in **Figure 1E**, after removal of nocodazole and regrowth of the MT network, mitochondria rapidly re-aggregated in BHRF1-expressing cells, indicating that an MT-dependent motor activity is involved in the mito-aggresome formation. Moreover, the hyperacetylation of MTs has been previously shown to facilitate the recruitment and binding of MAPs on the tubulin (Dompiere *et al.*, 2007). We therefore hypothesized that BHRF1-induced mitochondrial aggregation next to the nucleus could use dyneins. To investigate this hypothesis, we inhibited dynein functions by overexpression of p50-dynamitin. The overexpression of one subunit of the dynactin complex (p50-dynamitin) disrupts it and results in the dissociation of dyneins from the MT network. We observed that the expression of

this construction totally prevents mitochondrial aggregation in BHRF1-expressing cells (*Figures 5A and 5B*). Moreover, the ability of BHRF1 to induce mitochondrial fission was decreased when dyneins were inhibited, as we observed a partial but significant restoration of mitochondrial fission parameters (AR and FF; *Figure 5C*).

To confirm the importance of dynein motors in the mito-aggresome formation, we treated cells with ciliobrevin-D, a specific inhibitor of dyneins, which blocks their gliding along MTs and their ATPase activity. As with overexpression of p50-dynamin, BHRF1 was not able to induce mito-aggresomes in cells treated with ciliobrevin-D (*Supplementary file 4B and 4C*). However, BHRF1 still induced a slight mitochondrial fission phenomenon (*Supplementary file 4B and 4D*). Altogether, these results demonstrate that fragmented mitochondria are aggregated next to the nucleus in a dynein-dependent manner.

MT hyperacetylation does not affect BHRF1-pro-autophagic activity, but is required for the first step of mitophagy induction

Our group previously demonstrated that BHRF1 first stimulates autophagy and then induces mitophagy, the selective removal of mitochondria (Vilmen *et al.*, 2020). Autophagy is a conserved cellular process allowing the removal and recycling of damaged or supernumerary cellular components. This process involves double-membrane vesicles, called autophagosomes, which fuse with lysosomes to degrade the content. Selective autophagy consists of the recognition of a specific cargo by a molecular receptor that links the cargo to the autophagosome for its removal (Green and Levine, 2014).

Several studies demonstrated the role of MTs during the autophagic process, notably during the formation of the autophagosomes (Geeraert *et al.*, 2010; Köchl *et al.*, 2006; Mackeh *et al.*, 2013). Thus, we investigated whether BHRF1 could stimulate autophagy dependent on MT hyperacetylation. The autophagic flux was studied in HeLa cells co-expressing α -tubulin K40A and BHRF1. We first analyzed by immunofluorescence the accumulation of LC3 (microtubule associated protein 1 light chain 3), a classical marker of autophagosomes in the presence or absence of chloroquine (CQ), an inhibitor of the autophagic flux. When non-acetyltable α -tubulin was expressed, BHRF1 induced a clear accumulation of LC3 dots in the cytoplasm, and even more after CQ treatment (*Figure 6A*). We quantified LC3 dots and we observed no difference when MTs were hyperacetylated or not (*Figure 6B and supplementary file 5A*). We confirmed the result by analyzing the accumulation of lipidated LC3 (LC3-II) by immunoblot and we observed an increase in LC3-II levels in BHRF1-expressing cells, independently of MT hyperacetylation (*Figure 6C and Supplementary file 5B*). This increase is

potentiated after CQ treatment. Therefore, we concluded that BHRF1 stimulates the biogenesis of autophagosomes and the autophagic flux independently of MT hyperacetylation.

Mitochondrial aggregation in the perinuclear region has been already documented as essential to the efficient removal of mitochondria by mitophagy (Lee *et al.*, 2010). Although autophagy stimulation occurs independently of MT hyperacetylation, we investigated whether BHRF1-induced mitophagy depends on tubulin hyperacetylation by evaluating the colocalization between mitochondria and autophagosomes in cells co-expressing α -tubulin K40A and BHRF1. When MT hyperacetylation was prevented, mitochondria did not colocalize anymore with autophagosomes (*Figure 6D*). The quantification of colocalization intensity, measured by Manders' coefficient, confirmed that mitochondrial sequestration in autophagic vesicles did not occur when the mito-aggresome formation was impeded (*Figure 6E*).

We then decided to investigate whether, conversely, autophagy was involved in BHRF1-induced MT hyperacetylation. We observed that inhibition of autophagy by treatment with 3-methyladenine (3-MA) or spautin-1 impeded the MT hyperacetylation (*Supplementary file 5C*).

Altogether, these results demonstrated that MT hyperacetylation was essential for the mitochondrial sequestration but not for the induction of autophagy. In fact, BHRF1 needs autophagy activation to induce MT hyperacetylation. Therefore, we uncovered a novel mechanism to counteract anti-viral immunity, involving the initiation of mitophagy through the induction of tubulin hyperacetylation by BHRF1.

Discussion

We previously demonstrated that EBV-encoded BHRF1 blocks the IFN response by modifying the mitochondrial dynamics and by stimulating autophagy (Vilmen *et al.*, 2020). Expression of this viral protein induces mitochondrial fission, followed by the relocalization of fragmented mitochondria close to the nucleus in the proximity of the centrosome (**Figure 1 and Supplementary file 1**). Mitochondria are usually driven over long distances along MTs by motor proteins, thanks to the Miro/TRAK adaptor complex (Kruppa and Buss, 2021). Here, we demonstrate that the MT network is hijacked by BHRF1 to ensure the mito-aggresome formation and to consequently block the antiviral innate immunity (**Figures 1 and 3**). We notably observed that disruption of the MT network impedes mitochondrial trafficking to the centrosome, suggesting a dynamic process. In accordance, our results indicate that BHRF1 requires the MT-based motor dynein, that typically move towards the minus-end of MT, to regroup mitochondria in the perinuclear region (**Figure 5 and Supplementary file 4**).

The cytoplasmic dynein complex contains a pair of identical heavy chains which comprises the MT-binding domain. Each heavy chain harbors a motor domain that belongs to the AAA+ superfamily. Additional subunits complete this complex, each of which functions as a dimer; these are the intermediate chain, light-intermediate chain and three types of light chain (TCTEX, DYNLL1 and Roadblock) (Janke and Magiera, 2020; Roberts *et al.*, 2013). It has been reported that DYNLL1 can interact with BCL2L1/BIM, a proapoptotic BH3-only protein expressed at the mitochondrial outer membrane, and can regulate its proapoptotic activity (Puthalakath *et al.*, 1999; Singh *et al.*, 2019). Interestingly, it has been shown that BIM binds to BECN1, a key regulator of autophagosome formation, linking BECN1 to DYNLL1 and MTs, and leading to autophagy inhibition (Luo *et al.*, 2012). We and others demonstrated that BHRF1 can stimulate autophagy through its interaction with BECN1 and block apoptosis through its interaction with BIM (Desbien *et al.*, 2009; Kvensakul *et al.*, 2010; Vilmen *et al.*, 2020). Therefore, we hypothesize that BIM could connect mitochondria to MTs by interacting with BHRF1 and the MT-trafficking apparatus. The formation of this complex could explain the mitochondrial movement toward the centrosome and participate in autophagy regulation.

In eukaryotic cells, MTs are generally built from 13 chains of α - β tubulin dimers known as protofilaments. Properties and behaviors of MTs can be modulated by numerous post-translational modifications on both α and β tubulin (Janke and Magiera, 2020; Janke and Montagnac, 2017). We observed that the expression of BHRF1 increases the acetylation of α -tubulin on the lysine in position 40 (K40) and that acetylated MTs are required for the mito-aggresome formation (**Figure 2**). Acetylation of α -tubulin subtly changes its structure and disrupts the interaction between adjacent α -tubulins of neighboring protofilaments (Eshun-Wilson *et al.*, 2019). The loss of this connection weakens

interactions between protofilaments, which facilitates their sliding and decreases the flexural rigidity of MTs, making them more resistant to mechanical bending-induced breakage and disassembly (Portran *et al.*, 2017).

The hyperacetylation of MTs can result from either activation of tubulin acetyltransferases or inhibition of deacetylases (Li and Yang, 2015). Knockdown of the acetyltransferase ATAT1 by siRNA impedes the acetylation of α -tubulin and the mito-aggresome formation upon BHRF1 expression (**Figure 4**). Therefore, our results demonstrate that BHRF1 induces MT hyperacetylation by activating ATAT1. The expression of the non-acetylatable K40A mutant form of tubulin confirmed that tubulin acetylation, and not just ATAT1 activity, is required for the mito-aggresome formation and sequestration of mitochondria in autophagosomes (**Figures 2 and 6**). We also investigated a potential inhibitory role of BHRF1 on tubulin deacetylases. However, our results showed that BHRF1 does not impact the global tubulin deacetylase activity of BHRF1-expressing cells, suggesting that activities of HDAC6 and SIRT2, the two main α -tubulin deacetylases, are not inhibited by BHRF1 (Hubbert *et al.*, 2002; North *et al.*, 2003).

Hyperacetylation of MTs in the presence of BHRF1 could directly allow the spatial arrangement of mitochondria to the centrosome but the precise role of acetylated MTs will need to be further investigated. Misawa *et al.* demonstrated that accumulation of acetylated α -tubulin by knockdown of SIRT2 results in an aberrant mitochondrial distribution in the perinuclear region (Misawa *et al.*, 2013), similar to what was observed with BHRF1. Moreover, acetylated α -tubulin is more abundant in stable MTs, which are particularly implicated in mitochondrial transport (Friedman *et al.*, 2010). Interestingly, it has been shown that MT hyperacetylation *in vitro* and in cells causes the recruitment of dynein and kinesin-1 to MTs, which notably stimulates anterograde and retrograde transport of organelles in neurons or epithelial cells (Dompierre *et al.*, 2007; Geeraert *et al.*, 2010). BHRF1-induced acetylation of α -tubulin could thus increase dynein recruitment to MTs, facilitating the traffic of fragmented mitochondria to the centrosome.

It is interesting to note that MT hyperacetylation induced by BHRF1 is not involved in mitochondrial fission, since expression of the non-acetylatable α -tubulin mutant K40A or the knockdown of ATAT1 have no impact on mitochondrial length or branching parameters (**Figure 2F and Figure 4E**). We previously demonstrated that BHRF1 increases mitochondrial fragmentation in a Drp1-dependent manner (Vilmen *et al.*, 2020). Moreover, BHRF1 increases the dephosphorylation of Drp1 in position Ser637, which is then recruited to mitochondria to allow mitochondrial fission (Vilmen *et al.*, 2020). In our context, the lack of direct involvement of acetylated MTs on mitochondrial fission could seem quite surprising, since it has been previously shown that MT hyperacetylation triggered by acute

hyperosmotic stress enhanced mitochondrial fission (Perdiz *et al.*, 2017). However, in the context of this acute stress, mitochondrial fission is associated with an increased level of Ser616 phosphorylation of Drp1, a different post-translational modification from the one observed upon BHRF1 expression. Implication of hyperacetylated-MT in the mitochondrial fission process may thus differ according to the type of cellular stress.

BHRF1 is a multifunction protein (*e.g.* anti-apoptotic and pro-autophagic) localized at both the ER and the mitochondrial membranes. Since mitochondrial fission occurs at contact sites between ER and mitochondria, ER-localized BHRF1 could be involved in the ER remodeling into tubules which enwrap mitochondria and initiate the constriction of mitochondrial membranes before the recruitment of Drp1 (Friedman *et al.*, 2011). Conversely, the impact of BHRF1 on MT acetylation and mitochondrial trafficking could be related to its mitochondrial membrane localization.

We observed that MT hyperacetylation is not necessary to induce autophagy upon BHRF1 expression, whereas inhibition of autophagy impedes MT acetylation (**Figure 6 and Supplementary file 5**). This could seem unexpected, since several studies have demonstrated the role of MT acetylation in autophagosome formation and maturation, and the autophagy flux is directly correlated to MT acetylation levels (Esteves *et al.*, 2019; Geeraert *et al.*, 2010; Xie *et al.*, 2010). For example, initiation of starvation-induced autophagy requires MT acetylation to recruit dynein and kinesin-1 and to allow JNK (c-Jun N-terminal kinase) activation, leading to BECN1 release (Geeraert *et al.*, 2010). Very recently, it has been shown that p27, a tumor suppressor which promotes autophagy in response to glucose starvation, stimulates MT acetylation by binding to and stabilizing ATAT1, to allow the delivery of autophagosomes in the proximity of the centrosome for efficient fusion with lysosomes (Nowosad *et al.*, 2021). However, MTs is not involved in autophagosome formation in basal autophagy (Mackeh *et al.*, 2013). Although BHRF1-induced MT hyperacetylation has no role in non-selective autophagy, we observed that it is essential for BHRF1 to induce mitophagy since, in the absence of α -tubulin acetylation, BHRF1 does not induce mitochondrial sequestration into autophagosomes (**Figure 6**). This suggests that formation of the autophagosomal membranes around mitochondria requires acetylated MTs.

Numerous strategies are used by viruses to counteract or evade the innate immune response. We describe in this study a novel mechanism of a viral immune escape. Indeed, we report that MT hyperacetylation induced by BHRF1 can block IFN production, via mitophagy stimulation. Several proteins expressed by different viruses, such as human parainfluenza virus type 3, influenza A virus (IAV), or Hantaan virus (HTNV), are known to control type I IFN response by stimulating mitophagy (Ding *et al.*, 2017; Wang *et al.*, 2019, 2020). The viral glycoprotein (Gn) encoded by HTNV translocates

to mitochondria, induces complete mitophagy which leads to MAVS degradation and subsequently impairs innate immune response (Wang *et al.*, 2020). PB1-F2 is a non-structural IAV-encoded protein constitutively localized to the mitochondrial inner membrane space (Yoshizumi *et al.*, 2014). At mitochondria, the interaction of PB1-F2 with LC3B and mitochondrial TUFM (Tu translation elongation factor) similarly leads to mitophagy induction, MAVS degradation and restrains type I IFN production (Wang *et al.*, 2020). Whether these pro-viral mechanisms depend on the tubulin acetylation level needs to be explored.

The role of MT-dependent mitochondrial movement has been previously described to be involved in the NLRP3 (NOD-like receptor family pyrin domain containing 3) inflammasome, a complex that also contributes to antiviral innate immunity by the secretion of pro-inflammatory cytokines, such as interleukin-1 β and 18 (Zhao and Zhao, 2020). Detection of a viral infection or a danger signal can trigger the formation of this inflammasome, which is comprises of NLRP3, the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), and the effector protease caspase-1. Mitochondrial dynamics and movement are involved in inflammasome activation, to bring closer the fraction of ASC which resides on the mitochondria to NLRP3, located in the ER under resting conditions. It has been reported that acetylated MTs and dyneins are essential for the relocalization of mitochondria in the perinuclear region and the inflammasome complex formation (Misawa *et al.*, 2013). However, several studies have also reported that the activation of mitophagy can preclude NLRP3 inflammasome formation by preventing the release of mitochondrial DNA and ROS (Mishra *et al.*, 2021; Zhong *et al.*, 2016). The viral protein PB1-F2 negatively regulates the NLRP3 inflammasome (Yoshizumi *et al.*, 2014), and this could be related to its ability to stimulate mitophagy (Wang *et al.*, 2020). The activity of BHRF1 on the inflammasome is currently unknown but EBV encodes an miRNA that targets the *NLRP3* mRNA (Haneklaus *et al.*, 2012).

To conclude, we demonstrated how BHRF1, which is expressed during the early stages of EBV infection and some latency programs associated with lymphomas (Fitzsimmons and Kelly, 2017), reduces innate immunity by manipulating the MT network. We propose a model (**Figure 7**) in which EBV exploits acetylated MTs to change mitochondrial morphodynamics, thereby draining fragmented mitochondria toward the centrosome, segregating them from IFN signaling pathways in mitophagosomes to block IFN production and create an optimal environment for virus replication. We previously showed that BHRF1 expression induces mitochondrial fission activation and subsequently autophagy stimulation (Figure 7). We report here that MT hyperacetylation is downstream of these events. Beyond EBV infection and latency, these findings identify for the first time a role for acetylated MTs in regulating IFN response and innate immunity against viruses.

Materials and methods

Antibodies

Primary antibodies used in this study included anti- β -actin (Merck Millipore, MAB1501), anti-BHRF1 (Merck Millipore, MAB8188), anti-c-Myc (Sigma-Aldrich, M4439), anti-HA (Santa Cruz Biotechnology, sc-9082), anti-IRF3 (Novus Biologicals, SD2062), anti-LC3 (MBL International, PM036 and M151-3B), anti-pericentrin (Sigma-Aldrich, HPA016820), anti-TOM20 (BD Biosciences, 612278), anti- α -tubulin (Sigma-Aldrich, T6199) and anti-acetyl- α -tubulin (Sigma-Aldrich, T6793). Secondary antibodies used in this study included Alexa Fluor 488 goat anti-rabbit, anti-mouse (Jackson ImmunoResearch, 111-545-003, 115-545-003), Alexa Fluor 555 goat anti-rabbit, anti-mouse (Life technology, A21428, A21424), Alexa Fluor 647 donkey anti-rabbit, anti-mouse (Life technology, A31573, A31571). Horseradish peroxidase (HRP)-labeled goat anti-mouse and anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (115-035-003, 111-035-003).

Cell culture and transfection

HeLa and HEK293T cells were cultured at 37°C under 5% CO₂ in Dulbecco's modified eagle medium (DMEM; Gibco, 41965-039) supplemented with 10% fetal calf serum (FCS; Dominique Dutscher, S181B-500).

DNA plasmid transfections were performed using Fugene HD transfection reagent (Promega Corporation, E2311) according to the manufacturer's protocol. One day prior to transfection, cells were seeded in 24-well plates and incubated at 37°C. On the day of transfection, DNA plasmids were dispersed in opti-MEM (Gibco, 31985-047) and Fugene HD was added to the mix (ratio DNA:Fugene equals 1:3). After incubation for 10 min at room temperature (RT), the transfection mix was added to the cells. Then cells were incubated at 37°C and 6 h post-transfection, DMEM supplemented with 10% FCS was added. Depending on the expression plasmids, cells were fixed 24 h or 48 h post-transfection.

Extinction of ATAT1 expression

To deplete ATAT1 in HeLa cells, we performed transfection of siRNA directed against ATAT1 encoding mRNA (or non-relevant siRNA as control). We used ON-TARGETplus siRNAs from Dharmacon as a pool of four siRNAs: 5-GUAGCUAGGUCCCGAUUA-3; 5-GAGUAUAGCUAGAUCCCUU-3; 5-GGGAAACUCACCAGAA CGA-3; and 5-CUUGUGAGAUUGUCGAGAU-3. The transfections of siRNA were performed using Oligofectamine reagent (Thermo Fisher Scientific; 12.252.011) according to the manufacturer's protocol and were repeated at days 1, 2, and 3 at a final concentration of 100 nM. At the end of day 3, transfection with BHRF1-HA was also performed using Fugene HD transfection reagent as described above. Cells were then used at day 4 for immunoblotting or immunofluorescence analyses.

In vitro tubulin deacetylation assay

Cell lysates of control cells or cells transfected with BHRF1-HA plasmids were prepared using 10 mM Tris buffer pH 8.0, supplemented with 1% Triton, 1 mM NAD⁺, and the protease inhibitor cocktail. Lysate aliquots containing 20 µg of proteins were mixed with 2 µg of soluble acetylated tubulin dimers purified from porcine brain (Mackeh *et al.*, 2014; Walker *et al.*, 1988) and adjusted to 50 µL with NAD⁺-containing PEM buffer (80 mM PIPES, 2 mM EGTA, 1 mM MgCl₂). After 1 h at 37°C, reactions were stopped by adding 4X SDS-PAGE sample buffer and immediately subjected to SDS-PAGE and Western blotting of acetylated and total α-tubulin.

Immunoblot analysis

Cells were lysed in lysis buffer (65 mM Tris, pH 6.8, 4% SDS, 1.5% β-mercaptoethanol, and a cocktail of protease/phosphatase inhibitors) and kept at 100°C for 10 min. Protein extracts were resolved on SDS-PAGE (12.5%) and electro-transferred onto a polyvinylidene difluoride membrane (Amersham, 10600002). After 1h of incubation in blocking buffer (PBS, 0.1% Tween20, and 5% BSA or nonfat dry milk), the membranes were probed overnight at 4°C with primary antibodies. Horseradish peroxidase-labeled antibodies were used as secondary antibody, and revelation was performed using the ECL detection system according to the manufacturer's instructions (Merck Millipore, WBKLS0500). Scanning for quantification of protein levels was monitored using ImageJ software. An anti-β-actin antibody was used to ensure equal loadings and normalize quantification.

Immunofluorescence analysis

For all indirect immunofluorescence, cells were cultured on glass coverslips in 24-well plates. Cell monolayers were washed with PBS and cells were fixed with paraformaldehyde (PFA; 4%) in PBS or methanol, depending on the antibodies. The cells were treated with PBS containing NH₄Cl (50 mM) for 10 min, permeabilized using PBS, Triton X-100 (0.2%) for 4 min, washed twice with PBS and then incubated for 1 h in PBS, gelatin (0.2%) supplemented with FCS (5%) for blocking. Then the cells were incubated for 1 h with the appropriate primary antibody diluted in PBS, gelatin (0.2%) at RT. Cells were washed three times with PBS and then incubated with the appropriate secondary antibody diluted in PBS, gelatin (0.2%) for 1 h at RT. After washing, the nuclei were counterstained with DAPI. Coverslips were mounted in Glycergel (Agilent Dako, C056330-2) and observed using a Nikon Eclipse 80i epifluorescence microscope (Nikon Instruments) or a Leica TCS SP8 X inverted confocal microscope (Leica, USA). Photographic images were resized, organized, and labeled using Fiji-ImageJ software or LAS AF Lite. Imaris software was also used to perform 3D reconstruction video. A specific procedure was performed for the colocalization study between mitochondria and autophagosomes. Permeabilization and blocking were performed by incubating the cells for 30 min

with PBS, saponin (0.075%; Sigma-Aldrich, 84,510), BSA (1%), and FCS (5%). Antibodies were diluted in PBS, saponin (0.075%), and BSA (1%). In addition, between each antibody incubation, cells were washed twice in PBS, saponin (0.075%), BSA (1%), once with PBS, saponin (0.037%), BSA (0.5%) and twice in PBS. Other steps were the same as described above.

Luciferase reporter assays to assess IFN- β promoter activity

HEK293T cells were cultured in 24-well plates and transfected for 24 h using Fugene HD, with plasmids encoding the IFN- β luciferase reporter (firefly luciferase; 100 ng), pRL-TK (renilla luciferase; 25 ng), p Δ RIG-I (2xCARD) (encoding a positive dominant form of RIG-I; 150 ng), plasmids expressing BHRF1-HA (or EV as control; 150 ng) and mCherry-Tub (WT or K40A; 150 ng). One day after transfection, cells were lysed and measurement of firefly and renilla luciferase activities was performed using the dual-luciferase reporter assay system (Promega Corporation, E1910) according to the manufacturer's protocol. Relative expression levels were calculated by dividing the firefly luciferase values by those of renilla luciferase and normalized to the control condition (without BHRF1-HA expression).

Microtubules depolymerization and repolymerization

To depolymerize MTs, HeLa cells were seeded directly onto glass coverslips and incubated one day before being transfected to express BHRF1. Twenty-four hours after transfection, cells were treated with 10 μ M nocodazole (Sigma-Aldrich, M1404) for 2 h at 37°C then 1.5 h at 4°C, to depolymerize the MT network. Cells were immediately PFA 4% fixed and then methanol-permeabilized (t = 0 h before repolymerization). MTs and mitochondria were visualized by immunostaining of α -tubulin and TOM20, respectively. To allow reassembly of the MT network, nocodazole was removed by repeated washes with PBS, and cells were left 1 h in warm complete medium (t = 1 h after MTs regrowth). Cells were then immunostained for BHRF1, TOM20, and α -tubulin.

Mitochondrial phenotype assessment

BHRF1-induced mito-aggregates were scored by measuring the CI of mitochondrial staining, as we previously described (Vilmen *et al.*, 2020), based on Narendra *et al.* study (Narendra *et al.*, 2010). We arbitrarily determined that a cell presents a mito-aggregate when the CI is above 0.4. The percentage of cells presenting a mito-aggregate was determined by counting at least 20 random cells in each condition from three independent experiments.

Mitochondrial morphology was assessed by measuring AR and FF, which are indicators of mitochondrial length and branching, respectively. These parameters were quantified as described before (Koopman *et al.*, 2006). Briefly, on Fiji-ImageJ software, images of mitochondria were convolved using the matrix developed by Koopman, then a threshold was applied to isolate mitochondria from background. Then cell outline was drawn and mitochondrial phenotype was assessed by the

implement analysis particle tool. The AR value is given by the software and corresponds to the ratio between the major and minor axis of the ellipsis equivalent to the mitochondria. The FF value is defined as $(P_{mit}^2)/(4\pi A_{mit})$, where P_{mit} is the length of mitochondrial outline and A_{mit} is the area of mitochondria. The mitochondrial fission parameters were determined by analyzing at least 15 random cells in each condition from three independent experiments.

Plasmids

We previously described the BHRF1-HA expression vector (Vilmen *et al.*, 2020), which was constructed from a pcDNA3.1 expression vector (EV) purchased from Invitrogen (V79020). The mCherry- α -tubulin WT-K40K control vector was kindly provided by Dr. R. Y. Tsien (Department of Pharmacology, Howard Hughes Medical Center, University of San Diego, USA) (Shaner *et al.*, 2004) and the mutated vector (mCherry- α -tubulin K40A) was a kind gift from Dr. F. Saudou (Institut Curie, Orsay, France) (Dompierre *et al.*, 2007). The plasmids Flag- Δ RIG-I (2xCARD) and pIFN- β -Luc were previously described respectively (Yoneyama *et al.*, 2004, 1996) and kindly provided by Dr. C. Lagaudrière (I2BC, Gif-sur-Yvette, France). The plasmid pRL-TK was purchased from Promega Corporation (E2241). The p50-dynamin construct was a gift from Dr. S. Etienne-Manneville (Institut Pasteur, Paris, France) (Etienne-Manneville and Hall, 2001).

Reagents

MitoTracker Red CMXRos was purchased from Life Technologies (M7512) and used at 100 nM for 30 min before cell fixation to stain the mitochondrial network. Phalloidin–Tetramethylrhodamine B isothiocyanate was purchased from Sigma-Aldrich (P1951) and used at 20 μ g/mL during 1 h to stain F-actin. DAPI was purchased from Thermo Fisher Scientific (D1306) and used at 300 nM to stain dsDNA (nuclei). Nocodazole was purchased from Sigma-Aldrich (M1404) and used at 10 μ M to disrupt the MT network. Ciliobrevin D was purchased from Sigma-Aldrich (250401) and used at 40 μ M overnight to inhibit cytosolic dyneins. Cytochalasin B was purchased from Sigma-Aldrich (C6762) and used at 10 μ g/mL for 30 min to disrupt F-actin. Autophagic flux was monitored by the addition of chloroquine (Sigma-Aldrich, C6628) at 50 μ M for 2 to 4 h. To inhibit autophagy, we used two different ways: cells were treated either with 3-MA (Sigma-Aldrich, M9281) at 5 mM for 8 h or by spautin-1 (Sigma-Aldrich, SML0440) at 20 μ M for 8 h.

Results quantification

The number of endogenous LC3 dots per cell was determined using Fiji-ImageJ software. All images were taken with the same microscope settings to allow for comparison. Images were converted into 8-bit. Cells' outlines were drawn and nuclei cropped to conserve only the cytoplasm. A thresholding of images was performed to detect only LC3 vesicles. Note that the value of the threshold was conserved

and used for each analyzed cell. Finally, the “analyze particles” plug-in was used to count the total number of LC3 dots per cell. For each condition, at least 25 cells were analyzed in three independent experiments.

The colocalization degree between mitochondrial staining and autophagosomes staining was assessed by measuring Manders split coefficient using the “colocalization threshold” plug-in on Fiji-ImageJ software. For each condition, 30 random cells were analyzed.

The proportion of cells presenting an IRF3 nuclear localization was determined by counting at least 50 random cells in each condition from three independent experiments.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM) and were analyzed with Prism software (GraphPad) by using Student’s t-test. P values less than 0.05 were considered statistically significant. Data from three independent experiments were analysed.

Acknowledgments

We would like to thank for her technical assistance Valérie Nicolas (Université Paris-Saclay, Châtenay-Malabry, France) from the MIPSIT cellular imaging facility. Also, the present work has benefited from the core facilities of Imagerie-Gif, (<http://www.i2bc.paris-saclay.fr>), member of IBISA (<http://www.ibisa.net>), supported by “France-BioImaging” (ANR- 10-INBS-04-01), and the Labex “Saclay Plant Science” (ANR-11- IDEX-0003-02). Finally, we thank Stephanie Straubel for critical reading of the manuscript.

Additional information

Funding

Funder	Grand reference number	Author
CNRS, Univ Paris-Sud, DIM MALINF Région IDF	N/A	Géraldine Vilmen
Agence Nationale de la Recherche	ANR-14-CE14-0022	Audrey Esclatine
The authors declare that the funders had no role in study design, data collection or interpretation, or the decision to submit the work for publication.		

Author contributions

DG, GV, DP, conception and design, investigation, formal analysis, writing original draft; EH, investigation, formal analysis; GB, formal analysis, writing original draft, VM, formal analysis, funding acquisition, supervision; CP, formal analysis, provided materials; ML, conception and design, formal

571 analysis, writing original draft, supervision; AE, conception and design, formal analysis, funding
572 acquisition, writing original draft, supervision.

573 Authors ORCIDs

574 Damien Glon : <https://orcid.org/0000-0001-8405-8805>
575 Guillaume Beauclair : <https://orcid.org/0000-0001-5323-5987>
576 Vincent Maréchal : <https://orcid.org/0000-0003-2689-6266>
577 Christian Poüs : <https://orcid.org/0000-0002-2502-7854>
578 Marion Lussignol : <https://orcid.org/0000-0001-7586-2041>

579 Supplementary files

- 580 • Supplementary file 1. BHRF1 localizes in proximity of the centrosome.
- 581 • Supplementary file 2. The actin network is not involved in the formation of BHRF1-induced mito-
582 aggresomes.
- 583 • Supplementary file 3. Expression of the non-acetylatable α -tubulin K40A does not impact
584 mitochondrial network.
- 585 • Supplementary file 4. Dyneins inhibition prevents BHRF1-induced mito-aggresomes.
- 586 • Supplementary file 5. BHRF1 stimulates autophagy when α -tubulin WT is expressed, and
587 autophagy is required to MT hyperacetylation.
- 588 • Supplementary file 6. Full-length western-blot.

589 Data availability

590 All data generated or analyzed during this study are included in this published article and its
591 supplementary information files.

592 References

- Arnoult D, Soares F, Tattoli I, Girardin SE. 2011. Mitochondria in innate immunity. *EMBO Rep* **12**:901–910. doi:[10.1038/embor.2011.157](https://doi.org/10.1038/embor.2011.157)
- Castanier C, Garcin D, Vazquez A, Arnoult D. 2010. Mitochondrial dynamics regulate the RIG-I-like receptor antiviral pathway. *EMBO Rep* **11**:133–138. doi:[10.1038/embor.2009.258](https://doi.org/10.1038/embor.2009.258)
- Chatel-Chaix L, Cortese M, Romero-Brey I, Bender S, Neufeldt CJ, Fischl W, Scaturro P, Schieber N, Schwab Y, Fischer B, Ruggieri A, Bartenschlager R. 2016. Dengue Virus Perturbs Mitochondrial Morphodynamics to Dampen Innate Immune Responses. *Cell Host Microbe* **20**:342–356. doi:[10.1016/j.chom.2016.07.008](https://doi.org/10.1016/j.chom.2016.07.008)
- De Vos KJ, Allan VJ, Grierson AJ, Sheetz MP. 2005. Mitochondrial Function and Actin Regulate Dynamin-Related Protein 1-Dependent Mitochondrial Fission. *Curr Biol* **15**:678–683. doi:[10.1016/j.cub.2005.02.064](https://doi.org/10.1016/j.cub.2005.02.064)
- Desbien AL, Kappler JW, Marrack P. 2009. The Epstein-Barr virus Bcl-2 homolog, BHRF1, blocks apoptosis by binding to a limited amount of Bim. *Proc Natl Acad Sci U S A* **106**:5663–5668. doi:[10.1073/pnas.0901036106](https://doi.org/10.1073/pnas.0901036106)

- Detmer SA, Chan DC. 2007. Functions and dysfunctions of mitochondrial dynamics. *Nat Rev Mol Cell Biol* **8**:870–879. doi:[10.1038/nrm2275](https://doi.org/10.1038/nrm2275)
- Ding B, Zhang L, Li Z, Zhong Y, Tang Q, Qin Y, Chen M. 2017. The Matrix Protein of Human Parainfluenza Virus Type 3 Induces Mitophagy that Suppresses Interferon Responses. *Cell Host & Microbe* **21**:538–547.e4. doi:[10.1016/j.chom.2017.03.004](https://doi.org/10.1016/j.chom.2017.03.004)
- Dompierre JP, Godin JD, Charrin BC, Cordelieres FP, King SJ, Humbert S, Saudou F. 2007. Histone Deacetylase 6 Inhibition Compensates for the Transport Deficit in Huntington's Disease by Increasing Tubulin Acetylation. *J Neurosci* **27**:3571–3583. doi:[10.1523/JNEUROSCI.0037-07.2007](https://doi.org/10.1523/JNEUROSCI.0037-07.2007)
- Eshun-Wilson L, Zhang R, Portran D, Nachury MV, Toso DB, Löhr T, Vendruscolo M, Bonomi M, Fraser JS, Nogales E. 2019. Effects of α -tubulin acetylation on microtubule structure and stability. *Proc Natl Acad Sci U S A* **116**:10366–10371. doi:[10.1073/pnas.1900441116](https://doi.org/10.1073/pnas.1900441116)
- Esteves AR, Palma AM, Gomes R, Santos D, Silva DF, Cardoso SM. 2019. Acetylation as a major determinant to microtubule-dependent autophagy: Relevance to Alzheimer's and Parkinson disease pathology. *Biochim Biophys Acta, Post-Translational Modifications In Brain Health And Disease* **1865**:2008–2023. doi:[10.1016/j.bbadis.2018.11.014](https://doi.org/10.1016/j.bbadis.2018.11.014)
- Etienne-Manneville S, Hall A. 2001. Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKC ζ . *Cell* **106**:489–498. doi:[10.1016/s0092-8674\(01\)00471-8](https://doi.org/10.1016/s0092-8674(01)00471-8)
- Fitzsimmons L, Kelly G. 2017. EBV and Apoptosis: The Viral Master Regulator of Cell Fate? *Viruses* **9**:339–373. doi:[10.3390/v9110339](https://doi.org/10.3390/v9110339)
- Fransson Å, Ruusala A, Aspenström P. 2006. The atypical Rho GTPases Miro-1 and Miro-2 have essential roles in mitochondrial trafficking. *Biochem Biophys Res Commun* **344**:500–510. doi:[10.1016/j.bbrc.2006.03.163](https://doi.org/10.1016/j.bbrc.2006.03.163)
- Frederick RL, Shaw JM. 2007. Moving Mitochondria: Establishing Distribution of an Essential Organelle. *Traffic* **8**:1668–1675. doi:[10.1111/j.1600-0854.2007.00644.x](https://doi.org/10.1111/j.1600-0854.2007.00644.x)
- Friedman JR, Lackner LL, West M, DiBenedetto JR, Nunnari J, Voeltz GK. 2011. ER Tubules Mark Sites of Mitochondrial Division. *Science* **334**:358–362. doi:[10.1126/science.1207385](https://doi.org/10.1126/science.1207385)
- Friedman JR, Webster BM, Mastronarde DN, Verhey KJ, Voeltz GK. 2010. ER sliding dynamics and ER–mitochondrial contacts occur on acetylated microtubules. *J Cell Biol* **190**:363–375. doi:[10.1083/jcb.200911024](https://doi.org/10.1083/jcb.200911024)
- Geeraert C, Ratier A, Pfisterer SG, Perdiz D, Cantaloube I, Rouault A, Pattingre S, Proikas-Cezanne T, Codogno P, Poüs C. 2010. Starvation-induced Hyperacetylation of Tubulin Is Required for the Stimulation of Autophagy by Nutrient Deprivation. *J Biol Chem* **285**:24184–24194. doi:[10.1074/jbc.M109.091553](https://doi.org/10.1074/jbc.M109.091553)
- Giacomello M, Pyakurel A, Glytsou C, Scorrano L. 2020. The cell biology of mitochondrial membrane dynamics. *Nat Rev Mol Cell Biol* **21**:204–224. doi:[10.1038/s41580-020-0210-7](https://doi.org/10.1038/s41580-020-0210-7)
- Green DR, Levine B. 2014. To Be or Not to Be? How Selective Autophagy and Cell Death Govern Cell Fate. *Cell* **157**:65–75. doi:[10.1016/j.cell.2014.02.049](https://doi.org/10.1016/j.cell.2014.02.049)
- Hancock WO. 2014. Bidirectional cargo transport: moving beyond tug of war. *Nat Rev Mol Cell Biol* **15**:615–628. doi:[10.1038/nrm3853](https://doi.org/10.1038/nrm3853)
- Haneklaus M, Gerlic M, Kurowska-Stolarska M, Rainey A-A, Pich D, McInnes IB, Hammerschmidt W, O'Neill LAJ, Masters SL. 2012. Cutting Edge: miR-223 and EBV miR-BART15 Regulate the NLRP3 Inflammasome and IL-1 β Production. *J Immunol* **189**:3795–3799. doi:[10.4049/jimmunol.1200312](https://doi.org/10.4049/jimmunol.1200312)
- Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A, Yoshida M, Wang X-F, Yao T-P. 2002. HDAC6 is a microtubule-associated deacetylase. *Nature* **417**:455–458. doi:[10.1038/417455a](https://doi.org/10.1038/417455a)
- Janke C, Magiera MM. 2020. The tubulin code and its role in controlling microtubule properties and functions. *Nat Rev Mol Cell Biol* **21**:307–326. doi:[10.1038/s41580-020-0214-3](https://doi.org/10.1038/s41580-020-0214-3)
- Janke C, Montagnac G. 2017. Causes and Consequences of Microtubule Acetylation. *Curr Biol* **27**:1287–1292. doi:[10.1016/j.cub.2017.10.044](https://doi.org/10.1016/j.cub.2017.10.044)

- Kaufmann T, Kukolj E, Brachner A, Beltzung E, Bruno M, Kostrhon S, Opravil S, Hudecz O, Mechtler K, Warren G, Slade D. 2016. SIRT2 regulates nuclear envelope reassembly through ANKLE2 deacetylation. *J Cell Sci* **129**:4607–4621. doi:[10.1242/jcs.192633](https://doi.org/10.1242/jcs.192633)
- Khanim F, Mackett M, Young LS, Dawson J, Meseda CA, Dawson C. 1997. BHRF1, a viral homologue of the Bcl-2 oncogene, is conserved at both the sequence and functional level in different Epstein-Barr virus isolates. *J Gen Virol* **78**:2987–2999. doi:[10.1099/0022-1317-78-11-2987](https://doi.org/10.1099/0022-1317-78-11-2987)
- Köchli R, Hu XW, Chan EYW, Tooze SA. 2006. Microtubules Facilitate Autophagosome Formation and Fusion of Autophagosomes with Endosomes: Role of Microtubules in AV Formation. *Traffic* **7**:129–145. doi:[10.1111/j.1600-0854.2005.00368.x](https://doi.org/10.1111/j.1600-0854.2005.00368.x)
- Koopman WJH, Visch H-J, Smeitink JAM, Willems PHGM. 2006. Simultaneous quantitative measurement and automated analysis of mitochondrial morphology, mass, potential, and motility in living human skin fibroblasts. *Cytometry Part A* **69A**:1–12. doi:[10.1002/cyto.a.20198](https://doi.org/10.1002/cyto.a.20198)
- Kruppa AJ, Buss F. 2021. Motor proteins at the mitochondria-cytoskeleton interface. *J Cell Sci* **134**:1–13. doi:[10.1242/jcs.226084](https://doi.org/10.1242/jcs.226084)
- Kvansakul M, Wei AH, Fletcher JI, Willis SN, Chen L, Roberts AW, Huang DCS, Colman PM. 2010. Structural Basis for Apoptosis Inhibition by Epstein-Barr Virus BHRF1. *PLoS Pathog* **6**:e1001236. doi:[10.1371/journal.ppat.1001236](https://doi.org/10.1371/journal.ppat.1001236)
- Lee AJ, Ashkar AA. 2018. The Dual Nature of Type I and Type II Interferons. *Front Immunol* **9**:2061–2070. doi:[10.3389/fimmu.2018.02061](https://doi.org/10.3389/fimmu.2018.02061)
- Lee J-Y, Nagano Y, Taylor JP, Lim KL, Yao T-P. 2010. Disease-causing mutations in Parkin impair mitochondrial ubiquitination, aggregation, and HDAC6-dependent mitophagy. *J Cell Biol* **189**:671–679. doi:[10.1083/jcb.201001039](https://doi.org/10.1083/jcb.201001039)
- Li L, Yang X-J. 2015. Tubulin acetylation: responsible enzymes, biological functions and human diseases. *Cell Mol Life Sci* **72**:4237–4255. doi:[10.1007/s00018-015-2000-5](https://doi.org/10.1007/s00018-015-2000-5)
- López-Doménech G, Covill-Cooke C, Ivankovic D, Halff EF, Sheehan DF, Norkett R, Birsa N, Kittler JT. 2018. Miro proteins coordinate microtubule- and actin-dependent mitochondrial transport and distribution. *EMBO J* **37**:321–336. doi:[10.15252/emboj.201696380](https://doi.org/10.15252/emboj.201696380)
- Luo S, Garcia-Arencibia M, Zhao R, Puri C, Toh PPC, Sadiq O, Rubinsztein DC. 2012. Bim inhibits autophagy by recruiting Beclin 1 to microtubules. *Mol Cell* **47**:359–370. doi:[10.1016/j.molcel.2012.05.040](https://doi.org/10.1016/j.molcel.2012.05.040)
- Mackeh R, Lorin S, Ratier A, Mejdoubi-Charef N, Baillet A, Bruneel A, Hamaï A, Codogno P, Poüs C, Perdiz D. 2014. Reactive Oxygen Species, AMP-activated Protein Kinase, and the Transcription Cofactor p300 Regulate α -Tubulin Acetyltransferase-1 (α TAT-1/MEC-17)-dependent Microtubule Hyperacetylation during Cell Stress. *J Biol Chem* **289**:11816–11828. doi:[10.1074/jbc.M113.507400](https://doi.org/10.1074/jbc.M113.507400)
- Mackeh R, Perdiz D, Lorin S, Codogno P, Pous C. 2013. Autophagy and microtubules - new story, old players. *J Cell Sci* **126**:1071–80. doi:[10.1242/jcs.115626](https://doi.org/10.1242/jcs.115626)
- Misawa T, Takahama M, Kozaki T, Lee H, Zou J, Saitoh T, Akira S. 2013. Microtubule-driven spatial arrangement of mitochondria promotes activation of the NLRP3 inflammasome. *Nat Immunol* **14**:454–460. doi:[10.1038/ni.2550](https://doi.org/10.1038/ni.2550)
- Mishra SR, Mahapatra KK, Behera BP, Patra S, Bhol CS, Panigrahi DP, Praharaj PP, Singh A, Patil S, Dhiman R, Bhutia SK. 2021. Mitochondrial dysfunction as a driver of NLRP3 inflammasome activation and its modulation through mitophagy for potential therapeutics. *Int J Biochem Cell Biol* **136**:106013–106021. doi:[10.1016/j.biocel.2021.106013](https://doi.org/10.1016/j.biocel.2021.106013)
- Narendra D, Kane LA, Hauser DN, Fearnley IM, Youle RJ. 2010. p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. *Autophagy* **6**:1090–1106. doi:[10.4161/auto.6.8.13426](https://doi.org/10.4161/auto.6.8.13426)
- North BJ, Marshall BL, Borra MT, Denu JM, Verdin E. 2003. The Human Sir2 Ortholog, SIRT2, Is an NAD⁺-Dependent Tubulin Deacetylase. *Mol Cell* **11**:437–444. doi:[10.1016/s1097-2765\(03\)00038-8](https://doi.org/10.1016/s1097-2765(03)00038-8)

- Nowosad A, Creff J, Jeannot P, Culerrier R, Codogno P, Manenti S, Nguyen L, Besson A. 2021. p27 controls autophagic vesicle trafficking in glucose-deprived cells via the regulation of ATAT1-mediated microtubule acetylation. *Cell Death Dis* **12**:1–18. doi:[10.1038/s41419-021-03759-9](https://doi.org/10.1038/s41419-021-03759-9)
- Pagliuso A, Cossart P, Stavru F. 2018. The ever-growing complexity of the mitochondrial fission machinery. *Cell Mol Life Sci* **75**:355–374. doi:[10.1007/s00018-017-2603-0](https://doi.org/10.1007/s00018-017-2603-0)
- Perdiz D, Lorin S, Leroy-Gori I, Poüs C. 2017. Stress-induced hyperacetylation of microtubule enhances mitochondrial fission and modulates the phosphorylation of Drp1 at 616 Ser. *Cell Signalling* **39**:32–43. doi:[10.1016/j.cellsig.2017.07.020](https://doi.org/10.1016/j.cellsig.2017.07.020)
- Perdiz D, Mackeh R, Poüs C, Baillet A. 2011. The ins and outs of tubulin acetylation: More than just a post-translational modification? *Cell Signalling* **23**:763–771. doi:[10.1016/j.cellsig.2010.10.014](https://doi.org/10.1016/j.cellsig.2010.10.014)
- Portran D, Schaedel L, Xu Z, Théry M, Nachury MV. 2017. Tubulin acetylation protects long-lived microtubules against mechanical ageing. *Nat Cell Biol* **19**:391–398. doi:[10.1038/ncb3481](https://doi.org/10.1038/ncb3481)
- Puthalakath H, Huang DCS, O'Reilly LA, King SM, Strasser A. 1999. The Proapoptotic Activity of the Bcl-2 Family Member Bim Is Regulated by Interaction with the Dynein Motor Complex. *Mol Cell* **3**:287–296. doi:[10.1016/S1097-2765\(00\)80456-6](https://doi.org/10.1016/S1097-2765(00)80456-6)
- Reed NA, Cai D, Blasius TL, Jih GT, Meyhofer E, Gaertig J, Verhey KJ. 2006. Microtubule Acetylation Promotes Kinesin-1 Binding and Transport. *Curr Biol* **16**:2166–2172. doi:[10.1016/j.cub.2006.09.014](https://doi.org/10.1016/j.cub.2006.09.014)
- Roberts AJ, Kon T, Knight PJ, Sutoh K, Burgess SA. 2013. Functions and mechanics of dynein motor proteins. *Nat Rev Mol Cell Biol* **14**:713–726. doi:[10.1038/nrm3667](https://doi.org/10.1038/nrm3667)
- Shaner NC, Campbell RE, Steinbach PA, Giepmans BNG, Palmer AE, Tsien RY. 2004. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat Biotechnol* **22**:1567–1572. doi:[10.1038/nbt1037](https://doi.org/10.1038/nbt1037)
- Shida T, Cueva JG, Xu Z, Goodman MB, Nachury MV. 2010. The major α -tubulin K40 acetyltransferase α TAT1 promotes rapid ciliogenesis and efficient mechanosensation. *Proc Natl Acad Sci U S A* **107**:21517–21522. doi:[10.1073/pnas.1013728107](https://doi.org/10.1073/pnas.1013728107)
- Singh PK, Weber A, Häcker G. 2018. The established and the predicted roles of dynein light chain in the regulation of mitochondrial apoptosis. *Cell Cycle* **17**:1037–1047. doi:[10.1080/15384101.2018.1464851](https://doi.org/10.1080/15384101.2018.1464851)
- Tang B. 2018. Miro—Working beyond Mitochondria and Microtubules. *Cells* **7**:18–24. doi:[10.3390/cells7030018](https://doi.org/10.3390/cells7030018)
- Vilmen G, Glon D, Siracusano G, Lussignol M, Shao Z, Hernandez E, Perdiz D, Quignon F, Mouna L, Poüs C, Gruffat H, Maréchal V, Esclatine A. 2020. BHRF1, a BCL2 viral homolog, disturbs mitochondrial dynamics and stimulates mitophagy to dampen type I IFN induction. *Autophagy* **17**:1296–1315. doi:[10.1080/15548627.2020.1758416](https://doi.org/10.1080/15548627.2020.1758416)
- Walker RA, O'Brien E. T, Pryer NK, Soboeiro MF, Voter WA, Erickson HP, Salmon ED. 1988. Dynamic instability of individual microtubules analyzed by video light microscopy: rate constants and transition frequencies. *J Cell Biol* **107**:1437–1448. doi:[10.1083/jcb.107.4.1437](https://doi.org/10.1083/jcb.107.4.1437)
- Wang K, Ma H, Liu H, Ye W, Li Z, Cheng L, Zhang L, Lei Y, Shen L, Zhang F. 2019. The Glycoprotein and Nucleocapsid Protein of Hantaviruses Manipulate Autophagy Flux to Restrain Host Innate Immune Responses. *Cell Reports* **27**:2075–2091.e5. doi:[10.1016/j.celrep.2019.04.061](https://doi.org/10.1016/j.celrep.2019.04.061)
- Wang R, Zhu Y, Ren C, Yang S, Tian S, Chen H, Jin M, Zhou H. 2020. Influenza A virus protein PB1-F2 impairs innate immunity by inducing mitophagy. *Autophagy* **1**–16. doi:[10.1080/15548627.2020.1725375](https://doi.org/10.1080/15548627.2020.1725375)
- West AP, Shadel GS, Ghosh S. 2011. Mitochondria in innate immune responses. *Nat Rev Immunol* **11**:389–402. doi:[10.1038/nri2975](https://doi.org/10.1038/nri2975)
- Xie R, Nguyen S, McKeehan WL, Liu L. 2010. Acetylated microtubules are required for fusion of autophagosomes with lysosomes. *BMC Cell Biol* **11**:89–100. doi:[10.1186/1471-2121-11-89](https://doi.org/10.1186/1471-2121-11-89)
- Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* **5**:730–737. doi:[10.1038/ni1087](https://doi.org/10.1038/ni1087)

- Yoneyama M, Suhara W, Fukuhara Y, Sato M, Ozato K, Fujita T. 1996. Autocrine Amplification of Type I Interferon Gene Expression Mediated by Interferon Stimulated Gene Factor 3 (ISGF3). *J Biochem* **120**:160–169. doi:[10.1093/oxfordjournals.jbchem.a021379](https://doi.org/10.1093/oxfordjournals.jbchem.a021379)
- Yoshizumi T, Ichinohe T, Sasaki O, Otera H, Kawabata S, Mihara K, Koshiba T. 2014. Influenza A virus protein PB1-F2 translocates into mitochondria via Tom40 channels and impairs innate immunity. *Nat Commun* **5**:4713. doi:[10.1038/ncomms5713](https://doi.org/10.1038/ncomms5713)
- Zhao C, Zhao W. 2020. NLRP3 Inflammasome—A Key Player in Antiviral Responses. *Front Immunol* **11**. doi:[10.3389/fimmu.2020.00211](https://doi.org/10.3389/fimmu.2020.00211)
- Zheng K, Jiang Y, He Z, Kitazato K, Wang Y. 2017. Cellular defence or viral assist: the dilemma of HDAC6. *J Gen Virol* **98**:322–337. doi:[10.1099/jgv.0.000679](https://doi.org/10.1099/jgv.0.000679)
- Zhong Z, Umemura A, Sanchez-Lopez E, Liang S, Shalapour S, Wong J, He F, Boassa D, Perkins G, Ali SR, McGeough MG, Ellisman MH, Seki E, Gustafsson AB, Hoffman HM, Diaz-Meco MT, Moscat J, Karin M. 2016. NF-κB Restricts inflammasome activation via elimination of damaged mitochondria. *Cell* **164**:896–910. doi:[10.1016/j.cell.2015.12.057](https://doi.org/10.1016/j.cell.2015.12.057)

Figure 1. BHRF1 induces the formation of mito-aggresomes in a microtubule dependent manner. (A-C) HeLa cells were transfected for 24 h with BHRF1-HA plasmid or with EV as a control. Mitochondria were labeled with MitoTracker Red CMXRos and cells immunostained for BHRF1. Nuclei were stained with DAPI. (A) Confocal images with insets (3X) on mitochondrial phenotype. The values of the mitochondrial CI are indicated on representative cells. Scale bars: 10 μ m, and 2 μ m for insets. (B) *Left*, assessment of mitochondrial aggregation by calculation of CI. *Right*, percentage of cells presenting a mito-aggresome ($n = 20$ cells). (C) Quantification of mitochondrial fission parameters, aspect ratio (AR; *left panel*) and form factor (FF, *right panel*). (D) Confocal images of HeLa cells transfected with BHRF1-HA plasmid (or EV) and immunostained for pericentrin and BHRF1 (*upper panel*) or mitochondria (*lower panel*). Nuclei were stained with DAPI. Scale bar: 20 μ m. Arrowheads show the centrosomal localization of pericentrin. (E-F) MT network depolymerization assay. (E) Representative images where BHRF1 expression is visualized by HA immunostaining (gray), the MT network by α -tubulin immunostaining (green), and mitochondria are immunostained with an anti-TOM20 antibody (red). The values of the mitochondrial CI are indicated on representative cells. Scale bars: 10 μ m, and 2 μ m for insets. (F) Percentage of BHRF1-HA-positive cells presenting a mito-aggresome ($n = 30$ cells) in cells treated with nocodazole and after MT regrowth. Data represent the mean $\pm$ SEM of three independent experiments. ** $P < 0.01$; *** $P < 0.001$ (Student's t-test).

A

C

D

E

F

Figure 2. BHRF1 stimulates microtubules hyperacetylation, a process essential for the mito-aggresome formation. (A) Representative images of HeLa cells expressing BHRF1-HA and immunostained for HA and α -tubulin (*upper panel*) or acetyl- α -tubulin (*lower panel*). Nuclei were stained with DAPI. Scale bar: 20 μ m. (B) *Left*, immunoblot analysis of BHRF1-HA, acetyl and total α -tubulin. β -actin was used as a loading control. *Right*, the histograms show the normalized ratios of acetyl- α -tubulin to total α -tubulin. (C-F) HeLa cells were co-transfected with plasmids encoding BHRF1-HA (or EV) and either mCherry- α -tubulin WT (K40K) or a non-acetyltable form (K40A). (C) Immunoblot analysis of BHRF1-HA, acetyl and total α -tubulin. (D) Confocal images with insets (3X). BHRF1-HA expressing cells were visualized with an anti-HA antibody and mitochondria were immunostained with an anti-TOM20 antibody. Nuclei were stained with DAPI. Scale bars: 10 μ m and 4 μ m for insets. Values of mitochondrial CI are indicated on representative cells. Images of control cells are presented in **Supplementary Figure 3**. (E) Quantification of CI and cells with mito-aggregates (n = 20 cells). (F) Quantification of mitochondrial fission parameters (n = 20 cells). Data represent the mean $\pm$ SEM of three independent experiments. ns = non-significant; * P < 0.05; ** P < 0.01; *** P < 0.001 (Student's t-test).

A

C

B

Figure 3. Hyperacetylation of α -tubulin is required for BHRF1 to block IFN. **(A)** Luciferase reporter assay on HEK293T cells co-expressing BHRF1 (or EV) and α -tubulin K40A (or WT). Activation of the IFN- β promoter was analyzed 24 h post-transfection. Firefly/renilla luciferase ratios were calculated and normalized to control conditions. **(B and C)** HeLa cells were co-transfected for 24 h with plasmids encoding BHRF1-HA (or EV), mCherry- α -tubulin K40A (or WT), and Δ RIG-I (2xCARD). **(B)** Confocal images. BHRF1-HA-transfected cells were visualized with an anti-HA antibody and cells were immunostained with an anti-IRF3 antibody. Nuclei were stained with DAPI. Scale bar: 20 μ m. Arrowheads indicate BHRF1-expressing cells without IRF3 nuclear localization. **(C)** Percentage of cells expressing mCherry- α -tubulin and presenting IRF3 nuclear localization (n = 50 cells). Data represent the mean $\pm$ SEM of three independent experiments. ns = non-significant; ** P < 0.01; *** P < 0.001 (Student's t-test).

A

B

C

D

E

Figure 4. Characterization of MT hyperacetylation mechanism induced by BHRF1. **(A)** Tubulin deacetylation assay. HeLa cells were transfected with BHRF1-HA plasmid (or EV) and lysed 48 h post-transfection. Cell lysates were incubated *in vitro* with porcine brain tubulin and NAD⁺. BHRF1-HA, acetylated and total α -tubulin were detected by immunoblot, and the histogram represents the normalized ratios of acetyl to total α -tubulin. **(B-D)** After knockdown of ATAT1, HeLa cells were transfected with BHRF1-HA (or EV) and lysed/fixed 24 h post-transfection. **(B)** Extinction of ATAT1 activity visualized by immunoblot analysis of acetylated and total α -tubulin. **(C)** Confocal images. Mitochondria were labeled with MitoTracker CMXRos, and cells were immunostained for acetyl- α -tubulin and HA. Nuclei were stained with DAPI. Values of mitochondrial CI are indicated on representative cells. Scale bar: 20 μ m. **(D)** Quantification of CI and cells with mito-aggregates (n = 20 cells). **(E)** Quantification of mitochondrial fission parameters (n = 20 cells). Data represent the mean $\pm$ SEM of three independent experiments. ns = non-significant; * P < 0.05; ** P < 0.01; *** P < 0.001 (Student's t-test).

Figure 5. Dynein-based transport is required for BHRF1 to aggregate mitochondria next to the nucleus. **(A-C)** HeLa cells were co-transfected with plasmids encoding BHRF1-HA (or EV) and p50-dynamitin-myc (or control) for 24 h and then fixed. **(A)** Confocal images. Mitochondria were labeled with MitoTracker CMXRos, and cells were immunostained for c-myc and HA. Nuclei were stained with DAPI. Values of mitochondrial CI are indicated on representative cells. Scale bar: 20 μ m. Images of cells expressing EV and p50-dynamitin-myc are presented in **Supplementary Figure 4A**. **(B)** Quantification of CI and cells with mito-aggresomes (n = 20 cells). **(C)** Quantification of mitochondrial fission parameters (n = 20 cells). Data represent the mean $\pm$ SEM of three independent experiments. ns = non-significant; * P < 0.05, ** P < 0.01; *** P < 0.001 (one-way ANOVA test).

A α -tubulin K40A

C

D

E

Figure 6. MT hyperacetylation is dispensable for BHRF1-pro-autophagic activity, but essential for mitophagy. **(A-C)** HeLa cells were co-transfected for 24 h with plasmids encoding BHRF1-HA (or EV) and mCherry- α -tubulin K40A (or WT), and were treated with CQ when indicated. **(A)** Confocal images. Cells were immunostained for BHRF1 and LC3 and nuclei were stained with DAPI. Scale bar: 10 μ m. Images of cells transfected with mCherry- α -tubulin WT are presented in **Supplementary Figure 5A**. **(B)** Quantification of LC3 dots in each condition (n = 30 cells). **(C)** Immunoblot analysis of LC3 expression. **(D and E)** HeLa cells were co-transfected with BHRF1 (or EV) and mCherry- α -tubulin K40A (or WT). Cells were immunostained for TOM20 and LC3, and nuclei stained with DAPI. **(D)** Confocal images with insets (6X) showing colocalization events (see arrows). Scale bars: 10 μ m and 5 μ m for insets. **(E)** Colocalization level (Manders split coefficient) between mitochondria and autophagosomes. Data represent the mean $\pm$ SEM of three independent experiments. ns = non-significant; * P < 0.05; ** P < 0.01; *** P < 0.001 (one-way ANOVA test).

Figure 7. Working model. EBV encodes a Bcl-2 homolog that dampens type I IFN induction thanks to MT hyperacetylation. BHRF1 induces Drp1-mediated mitochondrial fission and subsequently autophagy. These cellular modifications lead to the mito-aggresome formation, and we showed here that the MT network and in particular its level of acetylation are essential for this mitochondrial phenotype. Moreover, BHRF1 induces MT hyperacetylation through ATAT1 activation. This hyperacetylation allows the retrograde transport of mitochondria in a dynein-dependent manner. Finally, the sequestration and degradation of mito-aggresomes block innate immunity.