# A fight against viral infections: host factors and antiviral therapies against positive-strand RNA viruses

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# Chapter 2

# Strategies employed by viruses to manipulate autophagy

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## Abstract

Autophagy, originally described as a conserved bulk degradation pathway important to maintain cellular homeostasis during starvation, has also been implicated in playing a central role in multiple physiological processes. For example, autophagy is part of our innate immunity by targeting intracellular pathogens to lysosomes for degradation in a process called xenophagy. Coevolution and adaptation between viruses and autophagy has armed viruses with a multitude of strategies to counteract the antiviral functions of the autophagy pathway. In addition, some viruses have acquired mechanisms to exploit specific functions of either autophagy or the key components of this process, the autophagy-related (ATG) proteins, to promote viral replication and pathogenesis. In this chapter, we describe several examples where the strategy employed by a virus to subvert autophagy has been described with molecular detail. Their stratagems positively or negatively target practically all the steps of autophagy, including the signaling pathways regulating this process. This highlights the intricate relationship between autophagy and viruses and how by commandeering autophagy, viruses have devised ways to fine-tune their replication.

**Keywords:** autophagy, viruses, virophagy, viral replication, antiviral immunity, ATG protein, autophagy receptor, hijacking, subvert

## The mechanisms of autophagy

### Microautophagy, macroautophagy and chaperone-mediated autophagy

The term autophagy derives from two Greek words that combinatorially mean self-eating, and groups all those intracellular delivering pathways that lead to the lysosomal degradation and recycling of intracellular components. There are three major types of autophagy: microautophagy, macroautophagy and chaperone-mediated autophagy (CMA) [1]. These three pathways deliver cytoplasmic cargoes into lysosomes through different mechanisms. Microautophagy involves the invagination of the lysosomal limiting membrane leading to a direct engulfment of cytoplasmic cargo by lysosomes [2]. In contrast, CMA requires the heat shock protein 70 (HSC70) chaperone protein to escort cytoplasmic proteins, mostly containing the conserved KFERQ pentapeptide, to a channel formed on the lysosomal surface by the lysosome-associated membrane protein (LAMP) type 2A for direct translocation into the interior of this compartment [2]. Macroautophagy, hereafter referred to as autophagy, is characterized by the formation of large double-membrane vesicles called autophagosomes that sequester the components destined for destruction in lysosomes [3].

### The molecular mechanism of autophagy and the autophagy-related (ATG) proteins

The entire process of autophagy can be subdivided in five sequential steps: initiation, elongation, maturation, fusion and degradation (Figure 1). Upon initiation of autophagy, autophagosome biogenesis begins with the formation of a membranous cistern known as the phagophore or isolation membrane. The phagophore subsequently elongates through the acquisition of extra lipids and the fusion of its extremities leads to the generation of the closed double-membrane autophagosome (Figure 1). The maturation step entails the disengagement and dissociation of the autophagy-related (ATG) proteins (see below) from the surface of autophagosomes and the recruitment of machinery responsible for fusion with lysosomes (Figure 1). The final steps comprise the fusion between the autophagosome and a lysosome and the subsequent exposure and degradation of the inner membrane of the autophagosome and the cargo by lysosomal hydrolases [4-7] (Figure 1).

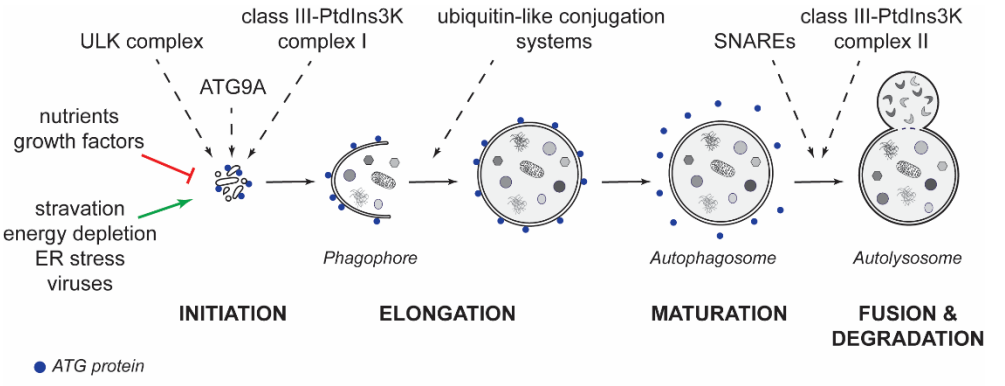


**Figure 1. Schematic model depicting the mechanism of autophagy.** The keys steps characterizing autophagy are initiation, elongation, maturation, fusion and degradation. Upon autophagy induction, the ULK complex, the class III PtdIns3K complex I and ATG9A catalyze the nucleation of the phagophore by probably mediating the heterotypic fusion of vesicles. Numerous signaling cascades regulating autophagy modulate the activity of these complexes. Thus, while nutrients and growth factors inhibit autophagy (red T-bar), nutrient starvation, energy depletion, ER stress and viral infections trigger this pathway (green arrow). The PtdIns3P generated on the surface of the phagophore is important for the recruitment of PtdIns3P-binding ATG proteins like WIPI2 and several components of the ATG12 and LC3 ubiquitination-like conjugation systems. Subsequently, these two ubiquitination-like conjugation systems drive the elongation and closure of phagophore to form the double-membrane autophagosome. The maturation step is marked by the release of ATG proteins and the turnover of PtdIns3P on the surface of the autophagosome. This is a pre-requisite for the engagement of the components of the machinery required for the fusion with endosomes and lysosomes, including SNARE proteins and other factor promoting this step such as the PLEKHM1 adaptor and the class III PtdIns3K complex II. After fusion, the autophagosomal cargo is degraded into metabolites by lysosomal hydrolases in autolysosomes.

The principal players orchestrating autophagosome biogenesis are the so-called ATG proteins. Sixteen of them compose the highly conserved ATG core machinery and they have been sub-grouped into five different functional clusters: (1) the ULK complex, (2) the autophagy-specific class III phosphatidylinositol 3-kinase (PtdIns3K) complex I (3) ATG9A and its binding partners and (4-5) two ubiquitin-like conjugation systems. The ULK complex is composed of ULK1 or ULK2 kinase, ATG13, RB1CC1/FIP200 and ATG101. This complex regulates the initiation of autophagosome formation by phosphorylating and interacting with several ATG components of the core machinery [4, 5] (Figure 1). The class III PtdIns3K complex I, which includes ATG14L, VPS34/PIK3C3, p150/PIK3R4 and BECLIN1 (BECN1), together with the ULK complex, is important for the phagophore nucleation and generation of phosphatidylinositol-3-phosphate (PtdIns3P) on autophagosomal membranes

(Figure 1). This lipid promotes the recruitment of PtdIns3P-binding proteins like WIPI1 and WIPI2 onto phagophores. The class III PtdIns3K complex I also directly interacts with proteins like AMBRA1 that are critical autophagy regulators [4, 8]. ATG9A is the only transmembrane protein within the core ATG machinery, and it functions in both phagophore formation and elongation. ATG9A shuttles between the trans-Golgi network/endosomes and phagophores upon autophagy induction, and this trafficking is regulated by both the ULK and PtdIns3K complexes. The two ubiquitin-like conjugation systems play a role in expansion and closure of the phagophore (Figure 1). The first ubiquitin-like conjugation system involves the covalent conjugation of ATG12 to ATG5, through the sequential action of the E1 activating enzyme ATG7 and the E2 conjugating enzyme ATG10. Subsequently, ATG16L1 binds non-covalently to the ATG12-ATG5 conjugate forming the ATG12-ATG5-ATG16L1 complex, which multimerizes through self-interaction. The second ubiquitin-like conjugation system begins with the cleavage of the members of the ATG8/LC3 protein family (i.e., LC3A, LC3B, LC3C, GABARAP, GABARAPL1 and GABARAPL2) by the cysteine proteases from the ATG4 protein family. This cleavage exposes a C-terminal glycine and this processed form of the LC3 proteins is referred to as LC3-I. Through the action of the E1 activating enzyme ATG7, the E2 conjugating enzyme ATG3 and guided by the ATG12-ATG5-ATG16L1 complex, LC3-I is covalently conjugated to the phosphatidylethanolamine (PE) molecules. LC3-I-PE is called LC3-II [4, 5].

Complete autophagosomes then tether, dock and finally fuse either first with endosomes and then lysosomes or directly with this latter organelle, to form autolysosomes (Figure 1). The mechanism of fusion is not completely understood, but it requires the RAB7 GTPase, the homotypic fusion and protein sorting (HOPS) complex, and adaptor proteins such as pleckstrin homology domain containing protein family 1 (PLEKHM1), and is driven by interaction of SNAREs, in particular SYNTAXIN17 (STX17) or YKT6, which are located on autophagosomes, and SNAP29, VAMP7, VAMP8 or VAMP9, on lysosomes [6, 7]. The PtdIns3K complex II, which differ from the autophagy-specific complex I in one subunit, i.e., UVRAG instead of ATG14L, has been shown to be important for fusion of autophagosome with lysosomes as well. UVRAG, through the binding to the HOPS complex, promotes RAB7 GTPase activity and stimulates the fusion between autophagosomes and lysosomes [6, 7]. The activity of UVRAG is negatively regulated by RUBICON, leading to the inhibition of the fusion between autophagosomes and lysosomes [6-8].

## The signaling cascades regulating autophagy

Autophagy initiation is mainly controlled through the regulation of two ATG functional clusters, the ULK complex and the class III PtdIns3K complex I, orchestrated by metabolic and stress sensors in a rapamycin complex 1 (mTORC1)-

dependent or -independent manner [3]. Now, we will present five main mechanisms regulating autophagy.



**Nutrient-sensing pathways**

Under nutrient-rich conditions, mTORC1 associates to and phosphorylates the ULK1 and ATG13 subunits of the ULK complex, leading to the inactivation of its kinase activity [3, 9]. In parallel and through phosphorylation, mTORC1 also inhibits the class III PtdIns3K complex I and the nuclear transport of the transcription factor EB (TFEB), a master transcriptional regulator of autophagy and lysosome biogenesis [3, 9]. Upon amino acid starvation, mTORC1 is inactivated and the factors modified by this kinase complex are dephosphorylated. This results in activation of both ULK and PtdIns3K complexes, triggering autophagosome biogenesis, and TFEB localization in the nucleus leads to the expression of genes involved in autophagy and lysosomal function [3, 4, 9].

MTORC1 activity is also regulated by the signaling cascades triggered by insulin and growth factors, two types of molecules that indirectly indicate the presence of nutrients (Figure 2A). Insulin and growth factors bind to their specific receptors, initiating a downstream signaling that leads to the activation of the class I PtdIns3K. The generated PtdIns3P enhances the activity of the protein kinase B (also known as AKT) [10], which inhibits TSC1/TSC2 complex through phosphorylation. mTORC1 activation requires GTP-bound Rheb. The TSC1/TSC2 complex acts as a GTPase-activating protein (GAP) for Rheb and consequently its inhibition allows the stabilization of GTP-bound Rheb and activation of mTORC1, which inhibits autophagy [9] (Figure 2A).

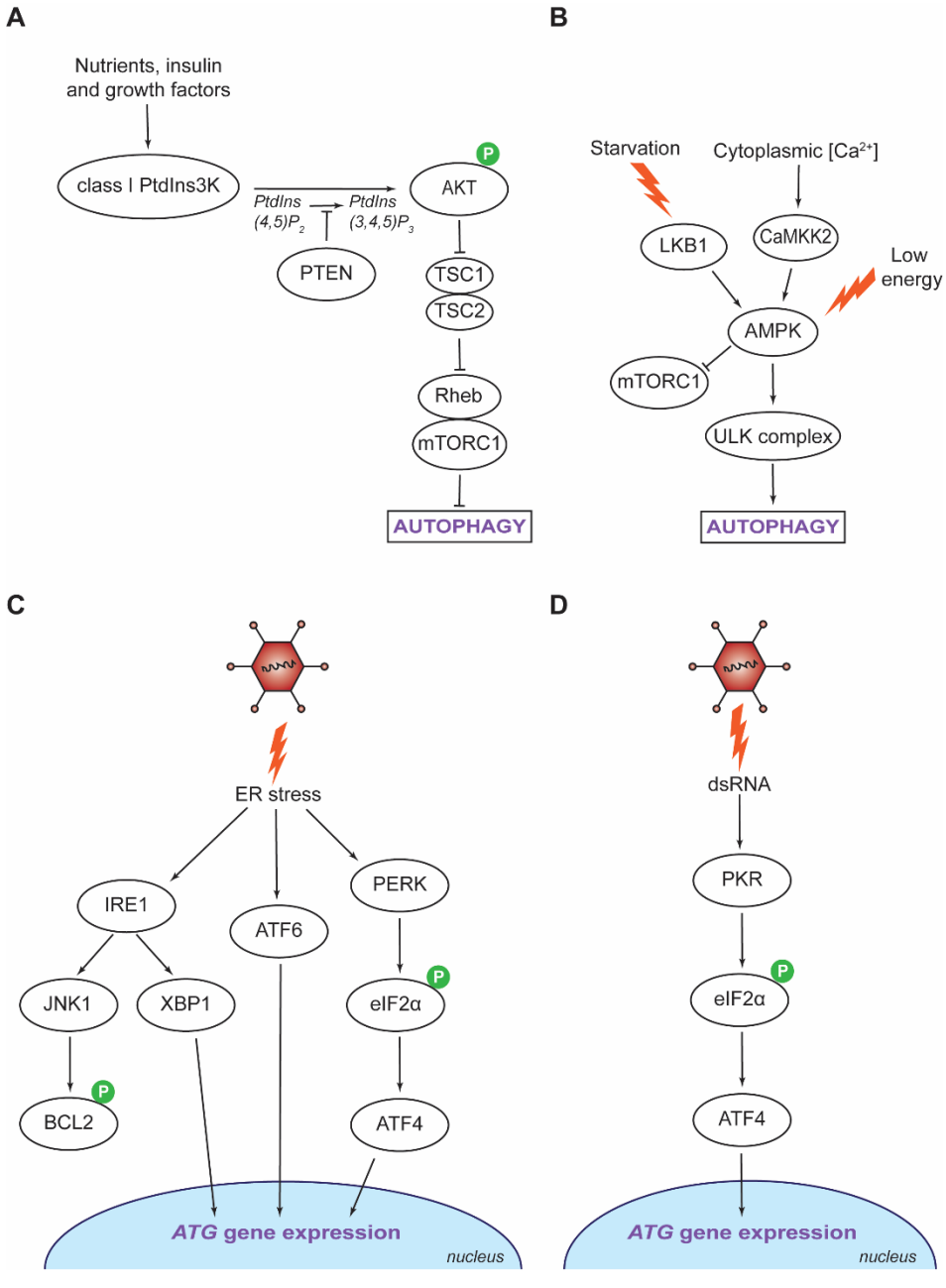


**Figure 2. The signaling cascades regulating autophagy.** (**A**) Orchestration of autophagy by mTORC1. This kinase complex acts as a major regulator of autophagy and numerous signals converge at this kinase complex, either activating or inhibiting autophagy. For example, the class I PtdIns3K/AKT/mTORC1 signaling axis inhibits autophagy through the activation of mTORC1. Nutrients or growth factors induces the AKT kinase, which inhibits TSC1/TSC2 complex through phosphorylation. TSC1/TSC2 acts as GTPase-activating protein for the GTP-

**Figure 2. (Continues from previous page).** bound Rheb. GTP-bound Rheb interacts and activate mTORC1 inhibiting autophagy. (**B**) Regulation of autophagy by AMPK. AMPK, which gets activated by either a decrease in the intracellular AMP:ATP ratio, LKB1 kinase or calcium-activated CaMKK2, positively regulates autophagy by inhibiting mTORC1 through phosphorylation of TSC1/TSC2 and RAPTOR. AMPK also directly phosphorylates components of the ATG machinery, such as the ULK complex and the class III PtdIns3K complex I, and stimulates them. (**C)** ER stress-induced autophagy. ER stress caused for example by virus infections triggers autophagy through the induction of the UPR. Autophagy can be stimulated by all the three branches that characterize the UPR, which are centered around IRE1, ATF6 and PERK, and allow cells to maintain ER homeostasis under stress conditions. IRE1 oligomerization leads to the alternative splicing of the XBP1 mRNA and the resulting translated transcriptional factor induces the expression of *ATG* genes. IRE1 also activates JNK, which phosphorylates BCL2 releasing inhibition from the class III PtsIns3K complex I. Cleaved ATF6 translocates into nucleus where this transcription factor triggers the expression of *ATG* genes. PERK activates eIF2α through phosphorylation. Phosphorylated eIF2α inhibits general cellular translation while promoting that of ATF4, a transcription factor that induces the transcription of *ATG* genes. (**D**) Triggering of autophagy by viruses. Presence of dsRNA leads to the activation of the PKR kinase, which phosphorylates and activates eIF2α triggering downstream signaling (see panel C).

Another axis regulating autophagy in response to stress conditions, is the one leading to the phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α). Various stress conditions, including amino acid deprivation, viral infection, ER stress and heme deficiency, causes the activation of distinct kinases, including the general control nonderepressible 2 (GCN2), the double-stranded RNA-activated protein kinase (PKR), the PKR-like endoplasmic reticulum-resident kinase (PERK), and the heme-controlled inhibitor (HRI). Phosphorylation of eIF2α leads to decrease in the global protein synthesis by decreasing the recognition and translation of mRNA with the AUG initiation codon, and favoring translation of specific mRNAs containing short ORFs, like the ATF4 transcription factor (Figure 2C). ATF4 subsequently drives the transcription and translation of many downstream proteins like SESTRIN2 and CHOP, which contributes to induction of autophagy by directly or indirectly inhibiting mTORC1 [3, 8].

Autophagy can also be modulated through the class III PtdIns3K complex I activity. In presence of nutrients, binding of B-cell lymphoma 2 (BCL2) to BECN1 inhibits the activity of this complex. Upon amino-acid starvation, BCL2 is phosphorylated by c-Jun N-terminal kinase (JNK1), triggering its dissociation from BECN1 and the concomitant class III PtdIns3K complex I activation [3, 8].

### Energy-sensing pathways

The AMP-activated protein kinase (AMPK) is a kinase that senses energy levels by detecting augmentations in the AMP/ATP ratio (Figure 2B). Binding of AMP to the γ-subunit of AMPK promotes its phosphorylation and activation by the upstream LKB1 kinase [11] (Figure 2B). Activated AMPK, also inhibits mTORC1 through phosphorylation. Additionally, AMPK phosphorylates and activates the TSC1/TSC2 complex, which is a negative regulator of mTORC1. Further, AMPK upregulates autophagy by directly phosphorylating and activating BECN1, VPS34 and ULK1 [9, 11].

### ER stress

The endoplasmic reticulum (ER) is a central cell organelle that fulfills a multitude of functions, including protein and lipid synthesis, protein folding and sorting, and calcium homeostasis maintenance. Environmental adaptations and pathophysiological processes that increase demand for protein synthesis and folding, disrupt protein folding leading to an accumulation of misfolded proteins, which in turn causes ER stress and the consequent activation of the unfolded protein response (UPR) [12]. UPR and autophagy are interconnected as autophagy alleviates ER stress by removing accumulated misfolded proteins (Figure 2C). The UPR comprises three distinct protein sensors, which initiates a downstream signaling upon activation by accumulation of misfolded proteins: PERK, inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) [12]. These three sensors positively regulate autophagy through different mechanisms (Figure 2C). PERK acts by phosphorylating eIF2α and triggering the downstream autophagy activation mechanism. Signaling downstream of IRE1 causes phosphorylation and activation of JNK1, which in turn phosphorylates BCL2 to release BECN1 and upregulate autophagy. In parallel, IRE1, through its endoribonuclease activity, yields a spliced variant of XBP1 that acts as a transcriptional activator of genes important for ER homeostasis but also for autophagy. ATF6 is activated by a cleavage event induced by ER stress. Cleaved ATF6 translocates into the nucleus and activates the transcription of genes important for ER homeostasis and autophagy [12, 13].

### Immune regulation of autophagy

Autophagy can be stimulated by many different immune signals, including interferon (IFN)-stimulated gene, PKR, toll-like receptors (TLRs), IFN-γ, stimulator of IFN genes (STING), immunity-related GTPase family M (IRGM), but also molecules such as tripartite motif (TRIM) proteins and the TANK-binding kinase 1 (TBK1) that have been linked to selective autophagy [14-17]. For example, the antiviral PKR kinase activates eIF2α-mediated autophagy upon sensing of dsRNA (Figure 2D). Recognition of pathogen-associated molecular patterns (PAMPs) by cellular pattern recognition receptors (PRRs) either at the cell surface or in the lumen of endosomes, also initiate downstream signaling that is mediated by myeloid differentiation primary response

protein 88 (MYD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF). These two adaptor proteins regulate the tumor necrosis factor receptor 6 (TRAF6) and TRAF3 ligases, which ubiquitinate the Lys63 residue of BECN1 and also promote the release of BCL2 from the class III PtdIns3K complex I. Moreover, damage associated molecular patterns (DAMPs) like DNA complexes, ATP and the high mobility group box 1 (HMGB1) protein can activate autophagy through AMPK-mediated mTORC1 inhibition or by releasing BCL2 from the class III PtdIns3K complex I [14, 17]. Multiple other cytokines have been shown to modulate autophagy, including TNF-α, IL-1, IL-2, IL-6 and TGF-β, which are positive regulators, and IL-4, IL-10, and IL-13, which are inhibitors [18]. Furthermore, IRGM which has been characterized as a genetic risk factor for Crohn's disease [19] and tuberculosis [20], has been shown to bind to ULK1 and BECN1, thereby promoting the orchestration of autophagy initiation complexes [21]. IRGM also interacts with several PRRs, including NOD1, RIG-I and TLR3, probably linking the innate immune response and autophagy [21].

### Autophagy and apoptosis

Autophagy has a dual role in cell survival and cell death. Autophagy-dependent cell death is a type of cell death requiring the ATG machinery for its execution [22]. During low levels of stress, cytoprotective function of autophagy tends to limit apoptosis by degrading damaged mitochondria or caspases, which are involved in intrinsic and extrinsic apoptosis and further supports cell growth by providing essential metabolites. Further, when stress exceeds beyond a certain threshold apoptosis is activated. Autophagy and apoptosis exhibit an extensive amount of cross talk [23, 24]. Autophagy and apoptosis share several regulatory upstream pathways and regulatory proteins. ATG proteins like BECN1, ATG3 can be cleaved by caspases, which inactivate their autophagic function and activate their pro-apoptotic functions. In addition, autophagy can also be regulated by several apoptotic proteins like BCL2 and caspase 8/FADD-like IL-1β-converting enzyme (FLICE)-inhibitory proteins (FLIP) to inhibit autophagy and proteins like BCL2 Interacting Protein 3 (BNIP3) and BNIP3-like (NIX) plays a proautophagic role. In addition, several upstream pathways like JNK and AKT also regulate both autophagy and apoptosis [24-26].

## The physiological functions of autophagy

Although autophagy was initially thought to be a bulk degradation process principally dedicated to the generation of an internal pool of nutrients in response of starvation, a multitude of recent studies has revealed the physiological relevance of the selective targeting of specific cargo by autophagosomes, a process referred to as selective autophagy. Selective autophagy can occur in ubiquitin-dependent or

-independent manner, and it requires the so-called autophagy receptors. Autophagy receptors associate to the cargo and mediate both the initiation of autophagosome biogenesis locally by interacting and recruiting the ULK complex, and the exclusive sequestration of the targeted cargoes into autophagosomes by binding the LC3 pool present in the interior of the expanding phagophore [27-30]. Some of the known autophagy receptors include p62/SQSTM1, NDP52, NBR1, TAX1BP1, OPTN and NIX/BNIP3. In addition, recent studies have highlighted that specific autophagy receptors such as OPTN, NDP52 and TAX1BP1 facilitate autophagosome maturation [31, 32].

Selective autophagy allows the specific removal of protein aggregates, dysfunctional or unnecessary complexes and damaged or superfluous organelles. Through these mechanisms, autophagy participates in numerous physiological processes such as adaptation to stress, programmed cell death, development and differentiation, and aging prevention [4, 33]. Not surprisingly, dysregulation of autophagy is associated with numerous diseases, including cancer development and progression, many neurodegenerative disorders like Alzheimer's, Parkinson's and Huntington's disease, and other age-related disorders [4, 33, 34].

Another key physiological function of autophagy is its role in eliminating cytoplasmic pathogens, including bacteria, virus and parasites, in a process termed xenophagy [16]. Here, innate immune receptors such as those belonging the TLRs family and autophagy receptors like p62, sense pathogens by recognizing various PAMPs and DAMPs, and host molecular tags such as ubiquitin and galectins, respectively, and guide autophagy in targeting microbes and/or microbe-related macromolecules to lysosomal degradation [16, 17].

In addition, autophagy also functions in controlling pro-inflammatory signaling [14, 17]. Finally, this pathway participates in multiple important aspects of the adaptive immunity, including MHC class II presentation of intracellular antigens, T cell survival and polarization, as well as T cell selection and maturation [14, 16, 17].

## Autophagy and viruses

Co-evolution between the host and pathogens has led microbes to develop different strategies to modulate autophagy to not only evade it, but also to subvert this pathway and the ATG machinery to successfully produce progeny particles. Here, we will summarize some of the strategies exploited by specific viruses to manipulate autophagy (Figure 3 and Tables 1 and 2). The interaction between autophagy and viruses can be either indirect, when viruses modulate upstream pathways regulating autophagy, or direct, by targeting ATG proteins or complexes (Figure 3).

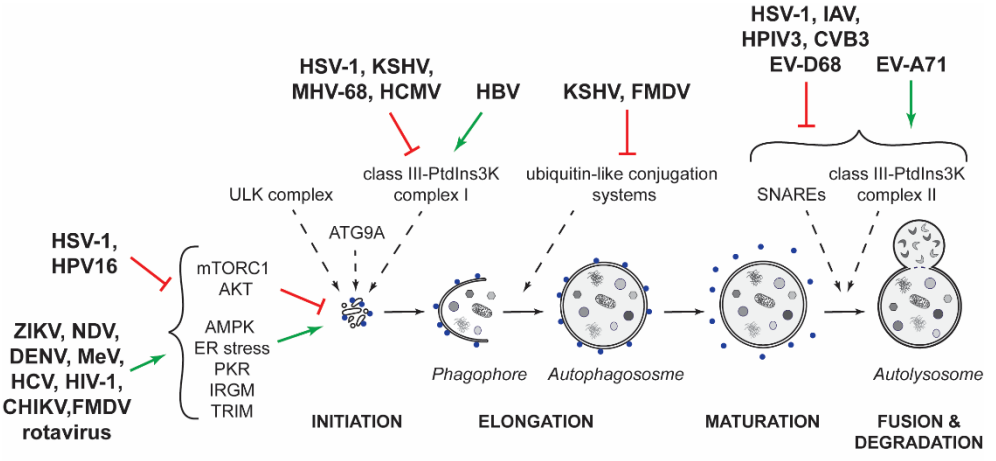


**Figure 3. Virus modulation of autophagy.** Viruses have developed numerous unique strategies to activate (green arrows) or inhibit (red T-bars) autophagy, by either modulating upstream signaling cascades or interfering with autophagosome formation and fusion with lysosomes through interaction with key players of the autophagy machinery. This scheme provides an overview of the viruses discussed in the review and the autophagy step that they subvert to promote their intracellular replication or cell egression.

## Table 1. Viral strategies to inhibit autophagic pathway

**Strategies to inhibit autophagic pathways**

| Virus family | Virus | Viral protein | Host target | Mechanism | References |
|---|---|---|---|---|---|
| *Strategies to inhibit autophagy by hijacking signaling pathways* | | | | | |
| *Herpesviridae* | HSV-1 | Us11 | PKR | Inhibition of PKR activity | [35] |
| | HSV-1 | Us11 | TRIM | Inhibition of TRIM- and TBK1-mediated autophagy | [36] |
| *Papillomaviridae* | HPV16 | ? | ? | Inhibition of the PI3K-AKT-MTOR signaling cascade by inhibiting PTEN | [37] |
| *Subversion of autophagy machinery* | | | | | |
| *Herpesviridae* | HSV-1 | ICP34.5 | BECN1 | Inhibition of the class III PtdIns3k complex I formation | [38] |
| | KSHV | vBCL2 | BECN1 | Inhibition of the class III PtdIns3k complex I formation | [39] |
| | MHV-68 | vBCL2 | BECN1 | Inhibition of the class III PtdIns3k complex I formation | [40, 41] |
| | HCMV | TRS1 and IRS1 | BECN1 | Inhibition of the class III PtdIns3k complex I formation and possibly also that of complex II | [42, 43] |
| | KSHV | vFLIP | ATG3 | Inhibit the LC3 conjugation system | [44] |
| *Picornaviridae* | FMDV | 3C Protease | ATG12-ATG5 conjugate | Degradation of the ATG12-ATG5 conjugate | [45] |
| *Disruption of selective types of autophagy* | | | | | |
| *Flaviviridae* | DENV, ZIKV | NS3 protease and NS2B | FAM134B | Cleavage and inactivation of FAM134B | [46] |
| *Picornaviridae* | CVB3 | 2A protease | p62 | Cleavage and inactivation of p62 | [47-49] |
| | CVB3 | 2A and 3C protease | NBR1 | Cleavage and inactivation of NBR1 | [47] |
| | CVB3 | 3C protease | NDP52 | Cleavage and inactivation of NDP52 | [49] |
| *The blockage of autophagosome-lysosome fusion* | | | | | |
| *Retroviridae* | HIV-1 | Nef | BECN1 | Speculated to modulate of the class III PtdIns3K complex II | [50] |
| *Orthomyxoviridae* | IAV | M2 | BECN1 | Speculated to modulate the class III PtdIns3K complex II | [51] |
| *Paramyxoviridae* | HPIV3 | P | SNAP29 | Interfering with SNAP29 function | [52] |
| *Picornaviridae* | CVB3 | 3C protease | SNAP29, PLEKHM1 | Cleavage and inactivation of SNAP29 and PLEKHM1 | [53] |
| *Picornaviridae* | EV-D68 | 3C protease | SNAP29 | Cleavage and inactivation of SNAP29 | [54] |

## Table 2. Viral strategies to induce autophagic pathway

**Strategies to induce autophagic pathways**

| Virus family | Virus | Viral protein | Host target | Mechanism | References |
|---|---|---|---|---|---|
| *Strategies to trigger autophagy by hijacking signaling pathways* | | | | | |
| *Flaviviridae* | ZIKV | NS4A and NS4B | ? | Inhibits AKT phosphorylation | [55] |
| *Flaviviridae* | DENV2 | ? | ? | IRE1α-JNK1 mediated phosphorylation of BECN1 | [56] |
| *Reoviridae* | Rotavirus | NSP4 | CaMKK2 | CaMKK2 mediated activation of AMPK-dependent autophagy | [57] |
| *Picornaviridae* | FMDV | VP1 | HSPB1 | Activation of ER stress-mediated autophagy via eIF2α-ATF4 cascade | [58] |
| *Flaviviridae* | DENV | ? | ? | Activation of AMPK-dependent lipophagy | [59] |
| *Paramyxoviridae* | Mev | C | IRGM | IRGM-mediated autophagy initiation | [60] |
| *Flaviviridae* | HCV | NS3 | IRGM | IRGM-mediated autophagy initiation | [60, 61] |
| *Retroviridae* | HIV-1 | Nef | IRGM | IRGM-mediated autophagy initiation | [60] |
| *Subversion of the ATG machinery* | | | | | |
| *Hepadnaviridae* | HBV | HBx | VPS34 | Enhancement of class III PtdIns3k complex I activity | [62] |
| | HBV | HBx | DAPK | DAPK-mediated activation of BECN1 | [63] |
| | HBV | HBx | BECN1 | Direct upregulation of BECN1 | [64] |
| | HBV | HBx | miR-192-2p | Inhibition of miR-192-2p, which negatively regulates BECN1 via XIAP | [65] |
| *Picornaviridae* | PV | ? | ? | ULK complex-independent autophagy activation | [66] |
| *The enhancement of autophagosome-lysosome fusion* | | | | | |
| *Picornaviridae* | EV-A71 | 2BC | STX17 | Unknown | [67] |
| *Paramyxoviridae* | Mev | ? | NDP52 and TAXBP1 | NDP52 and TAX1BP1 - mediated autophagosome maturation | [68] |

## Strategies to inhibit autophagy

### The hijacking of signaling pathways



Autophagy induction as a consequence of viral infection was first reported in a study involving herpes simplex virus 1 (HSV-1), which belongs to the *Herpesviridae* family (*Alphaherpesvirinae* subfamily) [69]. Specifically, the IFN□-inducible antiviral recognition pathway through PKR and eIF2α was found to upregulate autophagy in response to HSV-1 infection (Figure 2D). In this study, Tallóczy and colleagues revealed that mouse embryonic fibroblasts (MEFs) that lack PKR or carry non-phosphorylatable mutants of eIF2α, are unable to induce autophagy in response to HSV-1 [69]. HSV-1 antagonizes PKR-mediated autophagy through its protein Us11, which directly interacts and inhibit PKR [35]. In particular, a carboxy-terminal region of Us11 was found to be crucial for the binding to PKR. Thus, HSV-1 inhibits eIF2α phosphorylation, which in turn leads to both an inhibition of autophagy upregulation and the translation of viral proteins [35]. In addition, HSV-1 Us11 was also recently reported to inhibit autophagy by disrupting TRIM23 and TBK1 mediated autophagy [36]. TRIM23 is a E3 ubiquitin ligase enzyme with a C-terminal ADP ribosylation factor (ARF) domain, which harbors a GTPase activity. Auto-ubiquitination of the ARF domain activates TRIM23 and TBK1-mediated selective autophagy [70-72]. The exact mechanism of TRIM23 subversion by Us11 remains to be identified but the binding of Us11 to the ARF domain of TRIM23 probably disrupts the activation of TBK1 by TRIM23 and ultimately autophagy induction [36].

Human papillomavirus type 16 (HPV16), a DNA virus belonging to the *Papillomaviridae* family, inhibits autophagy during viral entry through activation of the cell surface epidermal growth factor receptor (EGFR) [37]. EGFR activation inhibits autophagy by inducing the class I PtdIns3K-AKT-mTORC1 signaling cascade (Figure 2A) [9, 10]. Binding of HPV16 virions to the cell surface causes a rapid AKT phosphorylation, which is mediated by EGFR as this does not occur when cells are treated with the specific EGFR inhibitor AG1478 [37]. This negative regulation of autophagy does not require the expression of viral proteins as the study used pseudo-virions, which are just capable of entering in the cells and deliver a pseudogenome to the cells without expressing viral proteins. In parallel, HPV also inactivates PTEN through phosphorylation of its Ser380 residue [73], which contributes to the inhibition of autophagy via class I PtdIns3K-AKT-mTORC1 signaling cascade (Figure 2A). Thus, incubation of keratinocytes with HPV16 for just 1 hour, caused a decrease in the LC3-II/I ratio, which may be indicative of autophagy suppression [37]. When autophagy is blocked by either treatment with 3-methyladenine (3-MA), class III PtdIns3K inhibitor, or knocking down of BECN1 or ATG7, infection was enhanced by 6 and 1.7-2-fold, respectively compared to the untreated control. Conversely, stimulation of autophagy by cell exposure to

tamoxifen, which is commonly used as an anti-cancer drug and a known autophagy inducer in cells expressing the estrogen receptor [74, 75], abrogated HPV16 infection [37]. These results show that HPV16 interferes with autophagy induction by manipulating EGFR-mediated signaling early in infection, which is ultimately beneficial for the infection outcome [37]. A subsequent study revealed that 3-MA enhances infection by protecting the HPV16 capsid from being targeted and degraded by autophagy [76].

**Subversion of the ATG machinery**

HSV-1 also inhibits autophagy initiation via the direct interaction of ICP34.5 with BECN1, which is a part of the class III PtdIns3k complex I [38]. Similar to BCL2 [77], binding of ICP34.5 to BECN1 appears to affect the formation of class III PtdIns3K complex I and prevents the generation of the PtdIns3P on autophagosomal membranes, which is necessary for the biogenesis of autophagosomes [38]. The interaction of ICP34.5 with BECN1 is mediated by its amino acids located at positions 68 to 87 and is independent of its GADD34 domain [38]. Studies in mice revealed that a mutant HSV-1 strain lacking 68-87 amino acids of ICP34.5 was severely neuroattenuated, because it leads to lower infectious titers and inflammation in the brain, resulting in a reduced animal mortality compared to control virus. This underlines the importance of the antiviral functions of autophagy, the crucial role of viral proteins in antagonizing autophagy and the impact that this has on disease pathophysiology [38].

Herpesviruses belonging to the *Gammaherpesvirinae* subfamily such as the Kaposi's sarcoma-associated virus (KSHV) and the murine γ-herpesvirus-68 (MHV-68), carry genomic BCL2 homologs to inhibit autophagy by binding to the BH3 domain of BECN1 [39-41]. Human cytomegalovirus (HCMV), which is part of the *Betaherpesvirinae* subfamily, encodes for two functional homologs of HSV-1 ICP34.5, called TRS1 and IRS1 [42, 43]. HCMV IRS1 binds to the coiled-coil region of BECN1, which is involved in binding to ATG14L1 and UVRAG [43, 78]. Thus, one plausible scenario is that IRS1 blocks autophagy by interfering with the formation of the class III PtdIns3K complex I and II. This and the fact that several viruses have acquired BCL2 homologues and/or target BECN1, suggests that there is a strong evolutionary pressure for them to be able to modulate autophagy and probably also apoptosis [24, 79].

In addition to BCL2, apoptosis is also regulated by several other proteins including FLIP, which negatively controls apoptosis by inhibiting caspase 8, but also autophagy through association to ATG3 [24, 80, 81]. FLIP association to ATG3 outcompetes the binding of LC3 proteins to this E2-like enzyme, and therefore blocks their conjugation to PE and ultimately autophagy. KSHV encodes a viral FLIP (vFLIP) and like the cellular FLIP, binds and inhibits ATG3 [44]. Cellular and viral FLIP proteins

contain two N-terminal domains called the death effector domain 1 (DED1) and 2 (DED2) [44]. Interestingly, 10 amino acids in the DED1 α2 helix and 12 in the DED2 α4 helix of vFLIP are sufficient to bind to ATG3. The α2 and α4 helixes do not only bind ATG3 but also cellular FLIP with high affinity and when expressed in cells, they can induce autophagy and autophagy-mediated cell death by blocking the interaction between endogenous FLIP and ATG3 [44]. The relevance of vFLIP-mediated inhibition of autophagy during KSHV infection, however, remains unknown.

Few studies have indicated that foot-and-mouth disease virus (FMDV), a member of the *Picornaviridae* family, induces autophagy during early stages of infection, which was monitored by detection of an initial increase in both the number of GFP-LC3 puncta and the levels of LC3-II [45, 58, 82]. Interestingly, a recent study showed that UV-inactivated FMDV is also able to trigger autophagy [58]. The authors of this study demonstrated that capsid protein VP2 stimulates autophagy by a mechanism involving eIF2α-ATF4 and AKT-mTORC1 signaling pathways. Specifically, VP2 appears to block heat shock protein beta-1 (HSPB1) activity, which causes an ER stress that leads to the activation of the eIF2α-ATF4 cascade (Figure 2C). Importantly, inhibition of autophagy by ATG5 depletion or 3-MA treatment significantly reduced FMDV genomic copies and titers [58]. However, a different investigation revealed that FMDV inhibits autophagy at the later time points of the infection by degrading ATG5-ATG12 complex using viral 3Cpro [45]. In line with this finding, overexpression of ATG12-ATG5 suppressed viral mRNA and protein synthesis, and viral progeny, whereas depletion caused an overall enhancement of FMDV infection [45]. The ATG12-ATG5 conjugate was suggested to positively regulate NF-κB and IRF3 antiviral signaling thereby inhibiting FMDV replication [45]. However, this is probably not linked to autophagy since the block of this pathway significantly reduces FMDV production [58] and therefore it could be attributed to an unconventional, non-autophagy related function of the ATG12-ATG5 conjugate.

### Disruption of selective types of autophagy

The role of selective autophagy in targeting viral proteins was first reported by Beth Levine's group by demonstrating that the selective autophagy receptor p62 targets Sindbis virus capsid protein for autophagic degradation [83]. Later, a similar phenomenon was shown to take place during Chikungunya virus infection [84]. As autophagy receptors are also regulating immunity [85], they represent an optimal target to simultaneously evade few host defenses.

The genome of flaviviruses, including Dengue (DENV) and Zika (ZIKV) virus, encode a NS3 protease and its cofactor NS2B [86]. The NS3-NS2B complex subverts reticulophagy, a selective type of autophagy leading to the turnover of ER, by

cleaving FAM134B, one of the specific reticulophagy receptors [46, 87]. Cleavage prevents FAM134B oligomerization [46], which is critical for ER membrane curvature and selective autophagic targeting [87, 88]. FAM134B cleavage occurs in the cytoplasmic loop at amino acid position 142. Mutation of this recognition site by changing the Arg into an Ala (i.e., FAM134BR142A), impairs FAM134B processing by the NS3-NS2B complex. Importantly, knockdown of FAM134B increases DENV and ZIKV replication, and their titers. Further, co-expression of cleavage-resistant FAM134BR142A variant together with the DENV NS2B3 complex resulted in an increase in the number of puncta positive for both FAM134B and NS3, suggesting that viral proteins are probably sequestered into reticulophagy-specific autophagosomes for degradation [46].

The autophagy receptor p62/SQSTM1 targets ubiquitinated proteins, complexes and organelles for autophagic degradation [89]. In addition to its role in autophagy, the N-terminal PB1 and TB domains of p62 are also involved in activation of NF-κB, which is key in mediating antiviral innate immune response and cell survival [90-92]. The protease 2Apro encoded by coxsackievirus B3 (CVB3), a picornavirus, cleaves p62 at Gly241. The resulting N- and C-terminal fragments are unlikely to sustain p62-mediated selective autophagy because contrary to the wild type p62, these fragments very poorly colocalize with LC3. Furthermore, these two fragments fail to facilitate NF-κB signaling compared to wild type p62 [48]. On this line, a recent investigation has demonstrated that p62 knockdown increases CVB3 viral titers [49]. Interestingly, the 2Apro and 3Cpro proteases of CVB3 cleave NBR1, and 3Cpro also NDP52 [47, 49]. Immunoprecipitation of p62 and NDP52 revealed that these two autophagy receptors interact with CVB3 capsid protein VP1, which was also ubiquitinated providing the possible underlying mechanism of VP1 recognition by p62 and NDP52 [47]. This study also showed that NDP52 has a CVB3 pro-viral role by suppressing type I IFN response through targeting the mitochondrial antiviral-signaling protein (MAVS) for autophagy-mediated degradation. Very interestingly, the authors demonstrated that although NDP52 is cleaved by viral 3Cpro, the resulting C-terminal fragment, which still contains the information to act as an autophagy receptor, retains this pro-viral role [49]. Further studies are required to determine whether this fragment has lost its capacity to target VP1 for virophagy. Of note, p62 is also processed by proteases encoded by other enteroviruses, including poliovirus, rhinovirus, and enterovirus D68 infection [54]. Thus, targeting selective autophagy receptors appears to be a common strategy adopted by enteroviruses to escape virophagy.

P62 has an antiviral role during DENV infection as well, as cell exposure to this virus leads to a time-dependent decrease in the levels of p62 while stable overexpression of this autophagy receptor results in a significant decrease in viral RNA and virus titers [93]. DENV, however, does not target p62 to lysosomal degradation but rather to proteasomal turnover [93].

**The blockage of autophagosome-lysosome fusion**

In primary human macrophages infected with the human immunodeficiency virus 1 (HIV-1), the Nef protein of HIV-1 was found to control autophagy by preventing fusion of autophagosomes with lysosomes [77]. Thus, Nef protects HIV-1 particles from degradation into autolysosomes. Interestingly, Nef co-localizes and co-immunoprecipitates with BECN1 and knockdown of BECN1 reduced virus yields [77]. Mutational analysis of Nef revealed that the di-acidic Asp motif at positions 174 and 175 interact with BECN1 [77]. Although Nef was initially also shown to interact with V-ATPase [78], a subsequent study demonstrated that Nef does not control the acidification of endosomes/lysosomes [79]. With recent studies showing the role of UVRAG-containing class III PtdIns3K complex II in autophagosome maturation [7], it is possible that Nef might prevent autophagosome-lysosome fusion by interfering with the formation of this complex. Nef may also interact with the RUBICON-positive PtdIns3K complex II, thereby increasing the inhibitory function of RUBICON on this complex [53]. In agreement with these data, another study showed that Nef expression in human astrocytes increases the accumulation of autophagy marker proteins such as LC3 and p62, confirming that Nef reduces the fusion of autophagosomes with lysosomes [80].

Similar to HIV-1, multiple Influenza A virus (IAV) strains were shown to inhibit fusion of autophagosomes with lysosomes as a cytoplasmic accumulation of autophagosomes was observed [51]. Ectopical expression of IAV matrix M2 protein was sufficient to cause an accumulation of autophagosomes and therefore the authors concluded that M2 is the protein responsible to interfere with autophagosome-lysosome fusion. Furthermore, silencing M2 or using a M2 knockout virus decreased autophagosome accumulation, thereby leading to an increased survival of IAV-infected cells. This suggests that a block in autophagosome-lysosome fusion promotes the IAV replication [51]. The M2 protein has a proton channel activity that is important for virion uncoating during virus cell entry [97, 98]. The proton channel activity of M2 is, however, not responsible for the inhibition of fusion as autophagosomes also accumulated in cells in presence of amantadine hydrochloride, an inhibitor of the proton channel [51, 97]. M2 co-immunoprecipitates with BECN1 and deletion of the first 60 N-terminal amino acids blocks this interaction [51]. Thus, similar to Nef, M2 might also target the PtdIns3K complex II, associated or not with RUBICON, to modulate the fusion of autophagosomes with lysosomes.

Human parainfluenza virus type 3 (HPIV3), a negative-strand RNA virus from the *Paramyxoviridae* family, also inhibits autophagosome-lysosome fusion but through a different mechanism [52]. Ectopic expression of the viral phosphoprotein (P) increased the cellular amounts of LC3-II and blocked fusion as tandem construct mCherry-GFP-LC3 [99] accumulated in yellow puncta, which represent cytoplasmic

autophagosomes [52]. To identify the mechanism by which P inhibits autophagosome-lysosome fusion, the authors performed a yeast two-hybrid screen and found that P specifically interacts with SNAP29 [52]. This was additionally validated by co-immunoprecipitation of the overexpressed proteins. The mechanism by which viral P protein interferes with autophagosome-lysosome fusion is by competitively binding of P to SNAP29, which prevents the binding of STX17 to SNAP29 which is important for fusion [52]. Importantly, induced accumulation of autophagosomes in HPIV3-infected cells through knockdown of SNAP29 or treatment with bafilomycin A1, resulted in an increase in extracellular viral particles without enhancing viral protein synthesis or intracellular viral load [52]. The authors of this study speculated a possible mechanism how inhibition of this step of autophagy could promote virion egression. Their supposition is based on the observation that the M protein of HPIV3 has a crucial role in the process of viral particle release from host cells [100] and treatment of cells expressing M protein with bafilomycin A1 increased the ability of M to bind to membranes [52]. This suggests that the accumulation of autophagosomes might increase the association of this viral protein with the plasma membrane. As a result, a plausible scenario is that the hijacking of the fusion machinery by HPIV3 might help in increasing M protein binding to the plasma membrane, which in turn promotes virus budding.

Coxsackievirus B3 also inhibits autophagosome-lysosome fusion as yellow puncta accumulated in infected cells expressing the mRFP-GFP-LC3 authophagic flux reporter whereas co-localization between LC3 and LysoTracker, a dye staining lysosome, was reduced [53]. The authors also showed an increase in the number of autophagosomes *in vivo* using a GFP-LC3 reporter mice [101], but whether this *in vivo* result is due to enhanced autophagosome formation or inhibition of fusion with lysosomes remains to be established [53]. *In vitro* downregulation of the expression of SNAP29, STX17, VAMP8 and PLEKHM1, in combination with the cleavage of SNAP29 and PLEKHM1 by the viral 3C protease was shown to destabilizes the SNARE complex resulting in autophagosome-lysosome inhibition [53]. Furthermore, knockdown of SNAP29 and PLEKHM1 increases the expression of viral capsid protein (VP1) and intracellular CVB3 titers, supporting the notion that autophogosomes might provide membranes for viral replication and/or allow CVB3 escaping xenophagy. Accumulation of autophagosomes might also play a role in virus release as CVB3 triggers the extracellular release of LC3-II-positive microvesicles containing infectious viral particles [102]. In this context, several investigations have described the role of unconventional secretory autophagy in non-lytic release of picornaviruses [102-105]. Thus, CVB3 may hijack the fusion machinery to favor the virus particle release [53]. Similarly, enterovirus D68 (EV-D68), another picornavirus, also inhibits the autophagic flux by 3C protease-mediated cleavage of SNAP29 [54].

Interestingly, Nowag and coworkers have revealed that Epstein-Barr virus (EBV), which belongs to the *Gammaherpesvirinae* subfamily, hijacks autophagy machinery

during its lytic cycle [106]. EBV has previously been shown to inhibit autophagic flux [107] and this study pointed out that EBV subverts the autophagy machinery to generate the envelope of viral particles [106]. Immunogold-labeling of electron microscopy of viral particle-enriched preparations showed the presence of lipidated LC3 in virus particles [106]. A similar finding has been also reported for HCMV, which initially triggers the formation of autophagosomes and then block their fusion with lysosomes [42, 108]. In particular, several ATG proteins were detected in the extracellular viral particles, including some of the LC3 proteins [108].

## Strategies to trigger autophagy

### The hijacking of signaling pathways

It has been shown that ZIKV induces autophagy in human fetal neural stem cells by interfering with AKT-mTORC1 pathway [55]. In particular, ZIKV replication leads to a reduction of AKT phosphorylation at Thr308 and Ser473, which are known to be modified by 3-phosphoinositide-dependent protein kinase 1 (PDK1) and mTORC2 [109-111], respectively. In turn, this causes a decrease in mTORC1 phosphorylation at Ser2448 that is followed by an induction of autophagy [55]. Co-expression of ZIKV non-structural proteins NS4A and NS4B also suppresses the AKT-mTORC1 signaling axis and triggered autophagy similar to ZIKV infection, showing that these two viral proteins are responsible for the subversion of this signaling pathway. Interestingly, NS4A and NS4B failed to induce autophagy upon expression of a constitutively active form of AKT, which confirmed that these two viral proteins interfere with the activation of AKT-mTORC1 cascade [55]. The increase of autophagy during ZIKV infection is beneficial for this pathogen as the knockout of ATG3 or ATG5, and the knockdown of ATG3 or ATG13, lead to a decrease in viral RNA and progeny virus production. The positive impact of autophagy on viral replication was also confirmed by treating cells exposed to ZIKV with several inducers and inhibitors of autophagy, including rapamycin, 3-MA and the lysosomal inhibitor chloroquine [55].

Viruses are known to induce autophagy via ER stress elicited by the accumulation of viral proteins in the ER that trigger the UPR [56, 112-115]. For example, initial studies on DENV infection have shown that autophagy induction is mediated by ER stress [116, 117]. A recent study by Lee and colleagues demonstrated the impact of ER stress-mediated autophagy on DENV serotype 2 (DENV2) replications both *in vitro* and *in vivo* [56]. Induction of ER stress and autophagy upon DENV2 infection was shown by an increase in the levels of both glucose-regulated protein 78kDa (GRP78), which is an ER chaperone and a marker for ER stress, and LC3-II. Alleviating ER stress using 4-phenyl butyric acid, which improves the ER folding capacity [118], showed a dose-dependent reduction in the cellular levels of GRP78 and LC3-II, and reduced viral protein NS1 expression and the extracellular viral titers [56]. The UPR PERK–eIF2α signaling axis was transiently activated at an early stage upon cell exposure to

DENV2 but had a minimal effect on autophagy. In contrast, IRE1 played a major role in the interplay between autophagy and DENV2 infection. Depletion of IRE1 using shRNA decreased the levels of LC3-II, viral NS1 and viral titers. IRE1 was shown to induce autophagy through JNK. DENV2 infection increases the levels of phosphorylated JNK in cells, which in turn phosphorylates BCL2, thereby releasing the BECN1-containing PtdIns3K complexes for autophagy activation (Figure 2C). These observations were further confirmed using SP600125, a specific inhibitor of JNK, which reduced the levels of phosphorylated JNK, phosphorylated BCL2, LC3-II and virus titers. An *in vivo* mice study revealed an induction in ER stress and autophagy upon DENV2 infection by a slight increase in the levels of GRP78 and LC3-II, respectively, in brain tissues. Additionally, inhibition of JNK signaling using SP600125 after inoculation of DENV2 reduced the LC3-II levels in the animal brain tissue similar to those in the control mice [56]. Further studies are needed to confirm the direct link between ER stress and autophagy by utilizing alternative assays to monitor autophagy progression, in order to determine whether the *in vivo* impact on virus infection of the JNK inhibitor is due to the regulatory role of this kinase in autophagy and not in other pathways such as apoptosis and inflammation [119], which might also contribute to the final positive outcome.

While DENV utilizes IRE1-JNK signaling axis during ER stress to induce autophagy [56], other viruses appear to use other stratagems. For example, the Newcastle disease virus (NDV) from the *Paramyxoviridae* family, exploits the PERK and ATF6 branches of the UPR (Figure 2C) with the same final beneficial effect for the virus replication cycle [114]. Chikungunya virus (CHIKV), which belong to the *Togaviridae* family, uses the IRE1-XBP1 signaling cascade to induce autophagy and limit cell death [120]. Thus, although many viruses exploit the UPR to modulate autophagy, different viruses seem to subvert different branches of the UPR.

Rotavirus, which belongs to the *Reoviridae* family, stimulates autophagy but blocks its flux [57]. This was shown by measuring an increase in the amounts of LC3-II and the number of LC3 puncta upon cell exposure to this virus, which did not increase upon cell treatment with bafilomycin A1. Furthermore, yellow puncta were amassed in infected cells expressing the tandem mRFP-GFP-LC3 reporter construct revealing an accumulation of cytoplasmic autophagosomes [57]. The same authors previously showed that rotavirus nonstructural protein 4 (NSP4) acts as a viroporin that releases calcium into the cytoplasm from ER [121]. It is well-known that an increase in cytoplasmic calcium leads to the activation of the resident calcium/calmodulin-dependent kinase kinase-β (CaMKK2), which in turn stimulates AMPK-dependent autophagy [122]. Interestingly, an NSP4 mutant protein with impaired viroporin activity was unable to induce autophagy, suggesting autophagy induction is mediated by higher levels of cytoplasmic calcium. [57]. This notion was confirmed by using a calcium chelator, which also inhibited autophagy induction by NSP4. Importantly, this investigation also showed that calcium-activated, AMPK-mediated

autophagy is important for the transport of NSP4 and the VP7 capsid protein from ER to the rotavirus replication/assembly sites as STO-609, an inhibitor of CaMKK2, blocked this event. The pharmacological and genetic impairment of autophagy or CaMKK2 inhibition decreased overall virus yield in host cells [57], indicating that autophagy hijacking for the traffic of specific viral proteins is important for the virus life cycle.

Several RNA viruses such as the measles virus (MeV), the hepatitis C virus (HCV) and HIV-1 induce autophagy by interacting with IRGM (Grégoire et al., 2011). Two studies reported that IRGM modulates autophagy by direct interaction with autophagy proteins such as BECN1, ATG5, ATG10, ATG14L1, ATG16L1, LC3C and ULK1 [21, 60]. Further, Chauhan and colleagues also reported that IRGM connects innate immune sensors like PRRs to autophagy [21]. Importantly, depletion of IRGM using siRNA and the consequent reduction of IRGM-mediated autophagy in response to the virus infection, caused a decrease in MeV, HCV and HIV-1 viral particle production to a similar extent as the ATG5 knockdown during infection [60]. The phenomenon is specific for these viruses as IAV was unaffected by IRGM knockdown. Furthermore, specific viral proteins, i.e., MeV C, HCV NS3 and HIV-1-Nef, directly interact with IRGM and are sufficient to trigger IRGM-mediated autophagy when ectopically expressed [60]. A recent study demonstrated that during HCV infection, IRGM positively regulates ULK1 activity by provoking its dephosphorylation at Ser757, which in turn stimulates ULK complex-dependent autophagosome biogenesis [61]. Altogether, these data show that RNA viruses belonging to different virus families hijack IRGM to induce autophagy and this leads to an increase in virus progeny [60]. It has been proposed that the increase in MeV virus production is associated with an autophagy-mediated protection against cell death [123]. In case of HCV, however, it has been proposed that autophagy plays a role in replication regulation [124-126], virus release by the exosomal pathway [127, 128] and inhibition of innate immune response [129, 130].

**Subversion of the ATG machinery**

So far there are not many examples showing subversion of the ATG machinery to promote viral infection. However, hepatitis B virus (HBV), which belongs so the hepadnavirus family, triggers the autophagic flux in multiple hepatoma cell lines but also in the liver tissues from HBV-infected patients [62]. As cell infection with this virus is not efficient, studies are often performed by transfecting HBV DNA into cells. Importantly, cell treatment with either 3-MA or siRNA targeting VPS34 or ATG7, inhibited HBV DNA replication. The multifunctional regulatory protein HBx encoded by HBV is responsible for autophagic flux induction as HBx mutant virus failed to stimulate this process. Interestingly, ectopically expressed HBx immunoprecipitated VPS34 indicating that HBx physically interacts with VPS34 and additionally these two factors colocalize shown by confocal microscopy. To determine whether HBx affects

the VPS34 activity *in vivo*, the authors of this investigation took advantage of a the p40phox-EGFP, which binds to PtdIns3P [131]. Co-expression of this reporter construct together with HBx showed an augmentation in GFP signal revealing that HBx might increase the intracellular PtdIns3P pool by enhancing the activity of one or more VPS34-containing class III PtdIns3K complexes [62]. A subsequent study, however, showed that ectopic expression of HBx inhibits autophagic degradation by impairing lysosomal functions [132]. It has additionally been shown that HBx induces autophagy by both direct up-regulation of BECN1 [64] and activation of the death-associated protein kinase (DAPK), which in turn phosphorylates and activates BECN1 [63]. A recent study has highlighted that HBx also stimulates autophagy by inhibiting miR-192-3p [65]. This microRNA negatively regulates X-linked inhibitor of apoptosis (XIAP), a factor that upregulates BECN1 levels through the activation of the NF-κB signaling pathway [65].

In addition to the HBV example, poliovirus (PV), which is part of the *Picornaviridae* family, could fall in the category of viruses that hijack the ATG machinery to promote viral infection. Previous studies have shown that PV infection induces autophagy and causes a rearrangement of the host membrane, which leads to the formation of double-membrane vesicles that are thought to being generated by the ATG machinery [133, 134]. A recent study by the same team has uncovered that autophagy, also shown by the formation of GFP-WIPI2 in infected cells, is triggered by PV in an ULK complex-independent manner, as LC3-II accumulated in ULK1- or FIP200-depleted cells infected with PV to the same extent as the control [66]. Moreover, double-membrane vesicle formation was also not influenced by the absence of FIP200 [66]. Additional studies, however, are needed to confirm that PV induces autophagy independently of the ULK complex by showing that under ULK and FIP200 knockdown conditions PV infection are still able to form GFP-WIPI2 puncta but not in the absence of other ATG genes. Although involving the viral proteins 2BC and 3A, the precise molecular details of the strategy employed by PV to trigger autophagy remains to be uncovered [135, 136].

### Hijacking selective types of autophagy

DENV2 subverts the AMPK signaling to induce lipophagy, the selective degradation of lipid droplets by autophagy [59]. In particular, DENV2 infection causes activation of AMPK signaling by increasing AMPKα-1 phosphorylation, which in turn inhibits of mTORC1 [59]. Silencing of AMPK or TSC2, reduces the total number of LC3-II positive puncta per cell and increases lipid droplets as a result of inhibition of DENV2 induced lipophagy. Importantly, DENV2-induced AMPK-mediated lipophagy is important for viral replication as silencing of AMPK or TSC2 also reduces viral RNA synthesis and titers. AMPK kinase activity is crucial for the viral replication cycle because a kinase-dead variant of AMPK did not complement the DENV2 replication defect of cells lacking wild type AMPK. However, the mechanism by which DENV2 activates the

AMPK signaling cascade remains unknown as well as the one used to selectively trigger lipophagy [59]. In addition, it remains to be determined whether the only currently known lipophagy receptor, the adipose triglyceride lipase (ATGL) [137], is critical for DENV2 infection.

A more recent study reported that bluetongue virus (BTV), which belongs to the *Reoviridae* family, evades IFN signaling by inducing the lysosomal degradation of signal transducer and activator of transcription 2 (STAT2) via autophagy, as inhibition of this pathway using 3-MA or by silencing ATG7 or BECN1 rescued STAT2 turnover [138]. Interesting, BTV-encoded NS3 binds and targets STAT2 for degradation [138]. As this event depends on NS3 ubiquitination and p62 co-localize with STAT2, a possible mechanistic scenario is that NS3 converts STAT2 into a substrate of selective autophagy.

As described above, some of the autophagy receptors like OPTN, NDP52 and TAX1BP1 facilitate autophagosome maturation [31, 32]. This function of the autophagy receptors appears to be exploited by MeV as the individual or combined knockdown of NDP52 and TAX1BP1 impaired the production of MeV infectious particles, suggesting that NDP52 and TAX1BP1-mediated autophagosome maturation seems to be crucial for MeV replication [68].

**Enhancement of autophagosome-lysosome fusion**

Opposite to CVB3 and EV-D68, enterovirus A71 (EV-A71), which also belongs to *Picornaviridae* family, enhances the formation of autolysosomes as cells exposed to this virus show an increase in both the autophagic flux and the number of mRFP-positive puncta when expressing the mRFP-GFP-LC3, compared to mock control [139]. The viral 2BC has been suggested to trigger autolysosome formation because its individual expression in cells lead to an accumulation of autolysosomes [139]. The mechanism by which 2BC increases fusion is unclear, but a yeast-two hybrid analysis uncovered an interaction between 2BC and STX17, which was validated by co-immunoprecipitation experiments in infected cells. This latter experimental approach also revealed that 2BC binds to SNAP29. Interestingly, immature viral capsid protein VP0 was also found to bind SNAP29 but the significance of this association remains to be explored. Enhanced autophagosome fusion with lysosomes might be beneficial for EV-A71 replication as inhibition of this step of autophagy by chloroquine, decreases viral protein and RNA synthesis, and viral titers [139]. This finding was confirmed by depleting different proteins involved in this fusion step, such as STX17, SNAP29 but also LC3B [140, 141], or the lysosomal protein LAMP1, which showed reduction in the ratio of mature/immature capsid proteins and virus titers [139]. Thus, autophagy, in particular autolysosomes formation, promotes EV-A71 replication and

possibly maturation of the viral proteins and/or the generation of infectious virions. How an enhanced autophagic flux can be beneficial for EV-A71, however, remains to be investigated.



## Conclusions and perspectives

Autophagy is a conserved catabolic pathway stimulated during cellular stress and is important to maintain cellular homeostasis by eliminating protein aggregates, damaged organelles and invading pathogens. Viruses have developed strategies to antagonize antiviral autophagy or ways to exploit this process for viral replication and progeny production. Antagonizing autophagy enables viruses to escape from degradation and in specific cases also limits cell death. In certain instances, it reduces cytokine production and immune signaling, which could be particularly crucial *in vivo*. Viruses that induce autophagy, on the other hand, appear to exploit autophagosome-derived membranes for replication or non-lytic virus particle release, but possibly also for transport of viral proteins between intracellular organelles.

Recent studies have started to highlight the intricate crosstalk between autophagy, ATG proteins, apoptosis, and inflammatory signaling. Frequently, viruses appear to subvert proteins that function as a regulatory hub with other cellular pathways such as BECN1, which limits cell death, or p62, which is also involved in autophagy-independent anti-viral signaling. Thus, although each virus has evolved its unique hijacking mechanism, many of them subvert the same component of the ATG machinery like BECN1, which is targeted to interfere with autophagy initiation, or SNAP29, which is modulated to alter fusion.

In spite of the growing number of studies showing that autophagy is induced or inhibited by many viruses in different cell types, there are relatively few investigations thoroughly pinpointing the molecular determinants and the mechanism behind the hijacking stratagem. Importantly, because autophagy is both a stress and immune response, it remains unclear whether the observed change in autophagy progression is directly caused by the virus or whether it is a consequence of the infection. In this context, it will be crucial to extend the cell culture-based studies to animal models to determine the relevance of autophagy subversion for viral replication and pathogenesis *in vivo*. There is a clear need to better characterize the molecular level of the interaction between ATG proteins and viral factors during specific infections. This knowledge will also be crucial to design precise experiments to determine whether the studied interactions are relevant for viral replication *in vivo* and therefore, also of therapeutic relevance.

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