

## STATE-OF-THE-ART REVIEW

# Escaping the Lion's Den: redirecting autophagy for unconventional release and spread of viruses

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## Keywords

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Autophagy is an evolutionarily conserved process, designed to maintain cellular homeostasis during a range of internal and external stimuli. Conventionally, autophagy is known for coordinated degradation and recycling of intracellular components and removal of cytosolic pathogens. More recently, several lines of evidence have indicated an unconventional, non-degradative role of autophagy for secretion of cargo that lacks a signal peptide. This process referred to as secretory autophagy has also been implicated in the infection cycle of several virus species. This review focuses on the current evidence available on the nondegradative features of autophagy, emphasizing its potential role and unresolved questions in the release and spread of (–) and (+) RNA viruses.

## Introduction

Viruses are obligate intracellular pathogens capable of directing cellular machinery for efficient viral genome replication and production of infectious progenies [1]. Viruses have co-evolved with their host to manipulate

## Abbreviations

ATG, autophagy-related protein; CDV, Canine Distemper Virus; CVB3, Coxsackie B3 virus; DENV, dengue virus; EBOV, Ebola virus; EV-D68, enterovirus D86; Gn or Gc, transmembrane glycoproteins; GRASPs, golgi reassembly and stacking proteins; HA, hemagglutinin; HCV, hepatitis C virus; HIV-1, human Immunodeficiency virus 1; HOPS, homotypic vacuole fusion and protein sorting; HPIV-3, parainfluenza virus type 3; HTNV, Hantaan virus; IAV, influenza A virus; IBV, Avian infectious bronchitis coronavirus; IRGM, immunity-associated GTPase family M; JEV, Japanese Encephalitis virus; LC3, microtubule-associated protein light chain 3; LDELS, LC3-Dependent Extracellular Vesicles Loading and Secretion; LIR, LC3-interacting region; M2, matrix-2; MARV, Marburg virus; MAVS, mitochondrial antiviral-signaling protein; MeV, Measles virus; MHV, mouse hepatitis virus; MVB, multivesicular Bodies; NS1, nonstructural protein 1; nsp6, nonstructural protein 6; PB2, polymerase basic protein 2; PE, phosphatidylethanolamine; PRRSV, respiratory syndrome virus; PV, poliovirus; RABV, rabies virus; RSV, respiratory syncytial virus; SARS-CoV, Severe and Acute Respiratory Syndrome virus; SFKs, Src family kinases; SNARE proteins, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; SNV, Sin Nombre Hantavirus; TEM, transmission electron microscopy; TUFM, mitochondrial Tu translation elongation factor; UPS, unconventional protein secretion; VLP, virus-like particle; VZV, Varicella Zoster virus; ZIKV, Zika virus.

various cellular processes. Autophagy is one such process that is heavily manipulated by both DNA and RNA viruses to support different stages of infection. Among others, RNA viruses are known to extensively remodel cellular organelles via manipulating various forms of autophagy to undergo replication and assembly [2].

Depending on the type of their genome, RNA viruses can be subdivided into negative-sense (−) and positive-sense (+) RNA viruses. A primary difference between them is that (−) viral RNA is first transcribed in the nucleus, whereas (+) viral RNA is directly translated in the cytoplasm [3]. Therefore, (+) RNA viruses are predominantly thought to exploit autophagy to generate replication and assembly sites in the cytoplasm, whereas (−) RNA viruses for viral protein transport. The role of autophagy specifically in the viral replication steps has been reviewed extensively before [4–6]. The focus of this review instead is on the involvement of autophagy in the lesser known process of RNA virus release and cell-to-cell spread.

Conventionally, release of (−) RNA virus progenies is thought to occur by budding of virions at the plasma membrane. The mechanism of (+) RNA virus release is less understood, but is presumed to occur via transport through the Trans-Golgi Network. However, emerging evidence indicates that RNA viruses have evolved mechanisms for unconventional secretion and spread. Unconventional viral release was first reported for Poliovirus, a nonenveloped enterovirus which was believed to spread solely by bringing about cell lysis. However, a role for autophagy was identified as virus-containing autophagic vesicles could be detected in the extracellular space and facilitated nonlytic viral spread [7,8]. Similarly, for human immunodeficiency virus (HIV), exosome-like vesicles containing virus or the Nef protein alone were detected in the extracellular space and were linked to increased infectivity and neurotoxicity [9,10]. Many more studies have implied that viruses can benefit from these autophagy-dependent unconventional secretion routes and a better understanding of how viruses hijack these egress strategies is of critical importance not only for fundamental insights into the viral life cycle but also to identify cellular factors that can be targeted to attenuate infection.

Autophagy is an evolutionarily conserved self-degradative process which is activated in response to stress and is critical for maintaining cellular homeostasis by removing misfolded or aggregated proteins as well as by eliminating damaged organelles and pathogens. Three major autophagy pathways have been described, chaperon-mediated autophagy, microautophagy, and macroautophagy. Here, we focus on the

exploitation of macroautophagy and hereafter refer to it as autophagy. Autophagy is characterized by the formation and maturation of a double membrane-bound vesicle, referred to as autophagosome, into a mature degradative autolysosome, involving broadly the following four complexes: (a) Unc-51-like kinase 1 (ULK1) complex, (b) activating class III phosphatidylinositol (PI3) kinase complex, (c) Atg5-Atg12-Atg16L complex, and (d) soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) autolysosome maturation complex (STX17-SNAP29-VAMP8 complex). Upon activation of the ULK1 complex, the PI3K complex is recruited to the endoplasmic reticulum to locally synthesize phosphatidylinositol and form the nascent autophagosomal cup. The Atg5-Atg12-Atg16L then acts akin to an E3 ligase to catalyze the transfer of phosphatidylethanolamine (PE) to the second ubiquitin-like system, microtubule-associated protein light chain 3 (LC3) to drive elongation of these membranes into a cargo-containing closed autophagosome [11,12]. The SNARE autolysosome maturation complex regulates autophagosomal fusion either with multivesicular bodies or late endosomes to form amphisomes or directly with lysosomes to form degradative autolysosomes [13–15]. Detailed mechanistic descriptions of degradative autophagy have been reviewed elsewhere [12–17].

As a defence mechanism, autophagy has been implicated in protecting the host from viral infection. For instance, Bcl2-associated athanogene 3 (BAG3) is a multifunctional HSP70 co-chaperone involved in selective autophagy, reported to negatively regulate Ebola virus (EBOV) and Marburg virus (MARV) budding. The viral matrix protein VP40 was sequestered away from the budding sites at the plasma membrane and incorporated into microtubule-associated protein light chain 3 (LC3)-containing aggresomes, thereby limiting virus release [18]. However, growing evidence indicates that viruses have co-evolved with the autophagy machinery to repurpose its function to promote production of virus progenies. Therefore, the type of interaction between specific viruses and the autophagy machinery is a key determinant of whether autophagy serves an antiviral or a proviral function.

Besides canonical degradative autophagy, viruses have been reported to exploit a lesser characterized arm of autophagy termed secretory autophagy. This is a nondegradative process that functions to sustain host cellular homeostasis by secreting proteins at the cell membrane via autophagosomes [19]. Proteins that lack the N-terminal signal peptide for canonical release via the Trans-Golgi network may be exported through this unconventional secretory autophagy pathway. In

secretory autophagy, the autophagosomes or amphisomes directly fuse with the plasma membrane rather than fusing with lysosomes for degradation. This process is otherwise referred to as noncanonical exocytosis. This mechanism was first reported for secretion of yeast acyl-coenzyme A-binding protein (Acb1) and has also been described for the unconventional protein release (UPS) of interleukin-1 $\beta$  (IL-1 $\beta$ ) [20,21]. IL-1 $\beta$  is a proinflammatory cytokine that lacks a signal peptide, which is required for conventional secretion via the trans-Golgi network. Instead, IL-1 $\beta$  is taken up in autophagic compartments of neutrophils during inflammation, via a mechanism that involves Golgi reassembly and stacking proteins (GRASPs), specific SNARE complexes, and Atg proteins. Upon knock-down of the early autophagy component Atg5, IL-1 $\beta$  secretion was significantly reduced, indicating IL-1 $\beta$  is released via these autophagic vesicles [22]. Furthermore for Acb1, it was shown that secretion of the Acb1-containing autophagosomes required MVB or endosomal fusion before release at the plasma membrane [20]. In a separate study, IL-1 $\beta$  was suggested to be secreted via 2 autophagic processes; (a) release in unilamellar, exosome-like vesicles after fusion of the autophagosome with the cell membrane or (b) vesicle-free release of virions at the cell membrane upon loss of the inner membrane in amphisome or lysosome acidification [23]. Similarly, secretory autophagy has been previously described to occur for enveloped DNA viruses like Varicella Zoster virus (VZV), that are indicated to exit the cells in autophagic membranes [24]. Moreover, recently Leidal *et al.* reported an additional unconventional secretion route for RNA binding proteins called LC3-dependent extracellular vesicles loading and secretion (LDELS), which may also be exploited by viruses. In this process, RNA binding proteins with an LIR motif are specifically captured by LC3II+ MVBs, which are subsequently packaged and released as extracellular vesicles upon MVB fusion with the plasma membrane [25]. This process therefore offers viruses the benefit of redirecting degradative autophagy toward secretion to support viral release in exosome-like vesicles or as free virions, that will be discussed for a selected set of RNA viruses in this review.

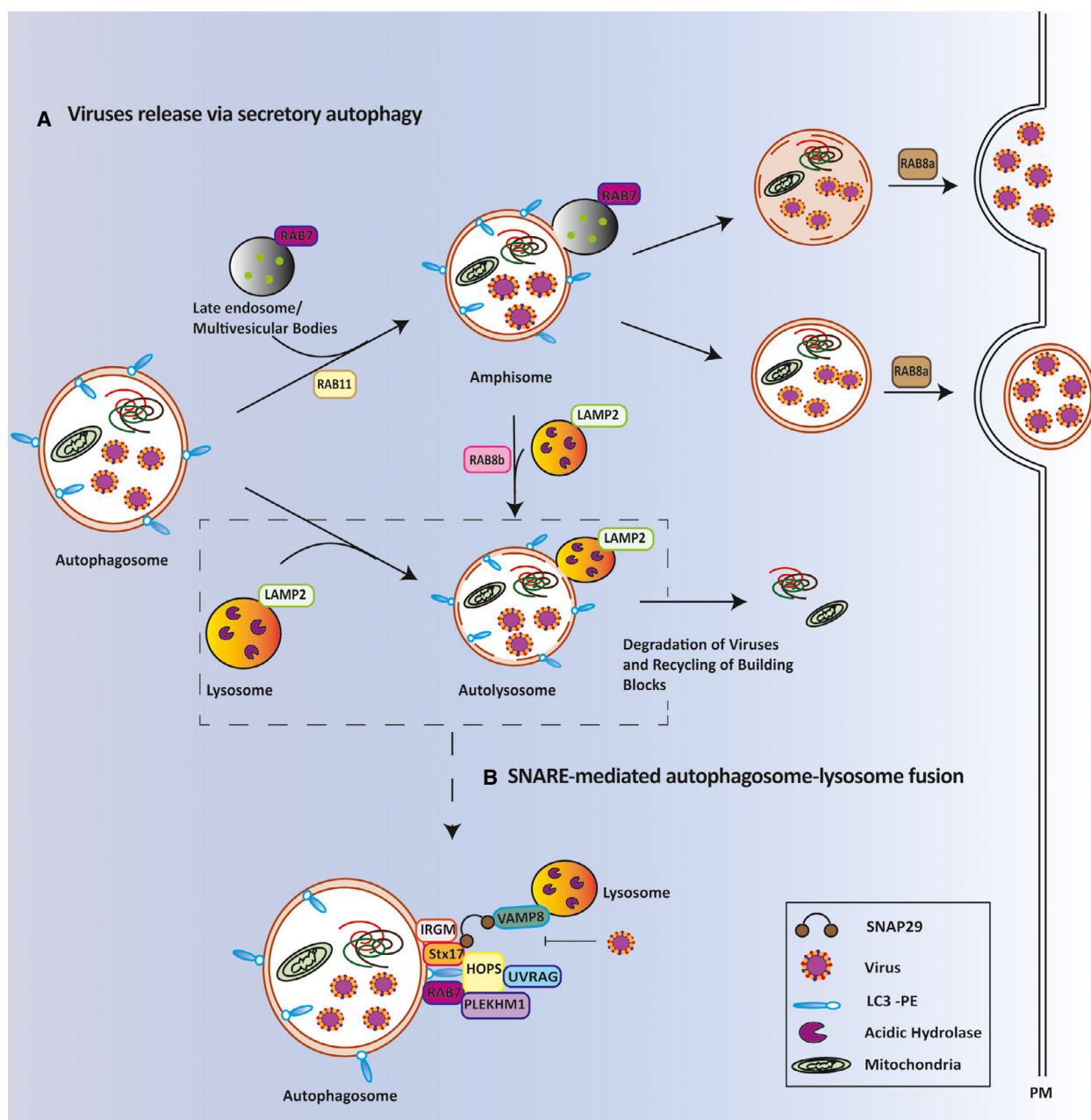
## Negative-strand RNA viruses

### Paramyxoviridae

The Paramyxoviridae family encompasses many viruses that are pathogenic in humans, such as Measles virus (MeV), parainfluenza virus type 3 (HPIV-3),

respiratory syncytial virus (RSV), and Rabies virus (RABV). HPIV-3 has been shown to subvert autophagy to enhance viral budding, by inhibiting fusion of the autophagosomes to lysosomes using its viral phosphoprotein (P). In addition to the Atg family, autophagosome maturation is regulated by SNARE complexes—STX17-SNAP29-VAMP8 (Fig. 1). The viral P protein binds to SNAP29, thereby hindering STX17 and VAMP8 from interacting with SNAP29 [26]. More remarkably, by employing virus-like particles (VLP), the authors observed that accumulation of autophagosomes enhanced release of viral matrix protein (M) and its colocalization with LC3 [26]. These results implied that autophagosomes could be redirected to the plasma membrane for viral budding. However, whether the M protein is incorporated within the autophagosomes to enhance autophagy-mediated virion budding or solely uses autophagosomes for transport is not known. In a follow-up study, the authors found that the interaction between the M protein and LC3 was a prerequisite for M protein to translocate to the mitochondria to induce mitophagy via its interaction with a mitochondrial Tu translation elongation factor (TUFM), resulting in dampened mitophagy-dependent type I Interferon (IFN) response [27]. Moreover, the study demonstrated that while autophagosome accumulation did not affect viral protein synthesis, it did increase extracellular viral yield [26], underscoring its involvement in virus release.

Depending on the virulence of the strain, Measles virus (MeV) is able to induce one or two successive waves of autophagy [28]. The first wave is transient and is induced upon virus binding to CD46, the MeV cell surface receptor [29]. The second wave of autophagy, reported to be sustained for longer, was shown to be required for efficient viral protein replication, innate response modulation, and virus production [30,31]. This later phase of autophagy was induced by MeV C protein in an immunity-associated GTPase family M (IRGM)-dependent manner [32]. IRGM was reported to interact with Atg5, Atg10, and LC3 and is commonly targeted by RNA viruses as a way to facilitate their life cycle [33,34]. Besides the MeV C protein, MeV infection-induced autophagy was shown to have limited impact on virus replication but was required for efficient cell-to-cell spread via LC3+ syncytia formation and membrane fusion [35]. Although the precise mechanism is not understood, it is tempting to speculate that the MeV C protein, that activates autophagy to facilitate formation of syncytia, is induced every time the syncytia fuses with a new uninfected cell, resulting in a positive feedback loop for induction of autophagy. Similar to MeV, membrane



**Fig. 1.** Schematic for virus release via secretory autophagy. (A) Upon completion of the isolation membrane, the autophagosome is transported to the perinuclear region of the cell in a RAB7-dependent manner. Here, autophagosome can fuse with multivesicular bodies (MVBs) or late endosomes forming an amphisome or fusing directly with lysosomes. Rather than RAB8b-dependent transport of these nascent amphisomes to lysosomes for degradation, the viruses can be alternatively released to the extracellular environment. Here, the amphisomes are transported to the plasma membrane (PM) for secretion in a RAB8a-dependent manner. At the PM virus progenies can be released directly, if the inner membrane has disintegrated or be released encapsulated in unilamellar vesicles. (B) Inhibition of SNARE-mediated autophagosome-lysosome fusion by viruses. The autophagosome-lysosome fusion is driven by the SNARE complex, comprising Stx17, SNAP29, and lysosomal VAMP8 to drive the fusion process. To complete autolysosomal fusion, HOPS and PLEKHM1 are recruited to autophagosomes by directly binding to LC3 and RAB7 as tethering factors. HOPS also binds to STX17 and is regulated by UVRAG. IRGM enhances recruitment of STX17 to autophagosomes and can bind LC3 directly. The components of the SNARE complex are commonly targeted by RNA viruses to exploit autophagy for release.



fusion is pivotal in inducing autophagy in canine distemper virus (CDV) infection, where activation of autophagy was found to facilitate viral dissemination by enhancing cell-to-cell fusion and syncytia formation in an Atg7-dependent manner [35].

### Bunyaviridae

Similar to many other (–) RNA viruses, members of the Bunyaviridae family have also been suggested to induce autophagy to restrain host innate immune response and promote viral replication. Bunyaviridae is an enormous family of over 350 RNA viruses divided into five genera. It is predominantly transmitted through arthropods; however, hantaviruses can occasionally transmit to humans through feces and urine of an infected rodent. Its virion structure encompasses a structural protein, nucleocapsid (N), two transmembrane glycoproteins (Gn and Gc), and L polymerase [36]. Gn and Gc are expressed from a single transcript and are then cleaved post-translationally into two glycoproteins. Hantaan virus (HTNV) has been shown to induce complete mitophagy at early stages of infection and incomplete autophagy at later stages. Similar to Sin Nombre Hantavirus (SNV), HTNV transmembrane glycoprotein Gn colocalizes with LC3-II and is selectively degraded through autophagic clearance [37]. Gn degradation at the early stage of infection is necessary for efficient viral production as demonstrated by knocking down Beclin-1 or Atg7 or by pharmacologically blocking selective degradation of Gn, both of which resulted in significantly decreased virus production [38]. Wang *et al.* showed that HTNV Gn interacts with TUFM at mitochondria to dampen type I IFN response through mitophagy and mitochondrial antiviral-signaling protein (MAVS) degradation. More remarkably, at later stages of infection, NP protein prevented Gn autophagic degradation by binding to LC3-II and SNAP29, to disrupt SNAP29-STX17 complex and block SNARE-mediated autophagosome maturation, thereby enhancing viral replication, assembly, and release [37]. HTNV thus induces a first wave of autophagy to escape immune sensing and an incomplete second wave of autophagy, which blocks viral protein degradation. NP-mediated inhibition of autophagy was shown to enhance HTNV release in a dose-dependent manner upon infection of NP-pretreated cells. How HTNV adopts opposing mechanisms of manipulating autophagy during infection needs further characterization, but it illustrates how diverse mechanisms are employed by these pathogens to hijack components of this pathway, even within a single life cycle.

### Orthomyxoviridae

The complexity of the interplay between autophagy and viruses is especially evident for the Orthomyxoviridae family and has been extensively studied in the context of influenza A virus (IAV) [28,39–42]. It should be noted that whether induction of autophagy during IAV infection is beneficial to the viral life cycle is still in dispute; however, it is generally thought that IAV induces incomplete autophagy to facilitate viral replication, assembly, and release. By employing a yeast two-hybrid array, Gregoire *et al.* identified several interactions between IAV viral proteins and autophagy-associated proteins, including polymerase basic protein 2 (PB2) with sequestosome 1 (SQSTM1) (human homolog p62), PB1-F2 with Atg5 and IRGM, Matrix-2 (M2) with Beclin-1, nonstructural protein 1 (NS1) with Atg5 and Golgi-associated PDZ and coiled-coil motif-containing protein (GOPC), NS2 with Atg5, Atg9A, IRGM, and UV radiation resistance-associated gene protein (UVRAG), and nucleoprotein (NP) with Atg5 and GOPC [33]. However, apart from the M2, the molecular mechanism involved in modulating autophagy by the remaining viral proteins is still to be elucidated. M2 is a proton-selective ion channel that is of vital importance for releasing the viral genome and associated proteins into the cytoplasm and in regulating virus assembly and budding [43]. Gannage *et al.* [44] reported that M2 is necessary and sufficient to block autophagosome maturation by binding to Beclin-1, resulting in the accumulation of small, high mobility autophagosomes, and formation of a single immobilized large perinuclear autophagosome. Notably, as a proton ion channel, whether the ability of M2 in inhibiting autophagosome maturation is independent from its proton ion channel activity is still contentious. Ren *et al.* [44,45] showed that M2 proton ion channel activity is necessary to inhibit fusion between autophagosomes and lysosomes, while Gannage *et al.* showed the opposite. On the other hand, Beale *et al.* [46] reported that IAV is able to subvert autophagy by interacting with LC3 through its M2 cytoplasmic tail, referred to as LC3-interacting region (LIR) to redistribute autophagosomes to the plasma membrane. Interestingly, mutations within this LIR region dramatically reduced filamentous virion budding and stability, thereby suggesting that IAV arrests autophagy to relocate LC3-containing autophagosomes to the plasma membrane to enhance viral proliferation [46]. Although LC3 is not integrated into the budding IAV virions, it appears that the presence of LC3 at the plasma membrane is able to modulate viral envelope composition by remobilizing appropriate

resources to the cell surface during budding, to assist in virion stability via unknown mechanisms. Apart from these mechanisms, Wang *et al.* [47] suggested another possible molecular mechanism for M2 to assist IAV in evading the autophagy machinery, by involving the host factor heat shock protein 90 $\alpha$  (HSP90AA1). HSP90AA1 is an essential chaperone that maintains PI3K/AKT activity by binding to AKT and interacting with PB2 to facilitate viral RNA synthesis, viral ribonucleoprotein (vRNP) nuclear import, and viral assembly [48–50]. The authors found that in addition to colocalizing and interacting with LC3 in the perinuclear region, which results in enhanced vRNP export and infectious virion formation, M2 and NP also increased HSP90AA1 expression and regulated the AKT-mTOR pathway to mediate autophagosome accumulation [47]. Therefore, at early stages of infection, NP and M2 induced autophagy to facilitate viral RNA synthesis via increased levels of HSP90AA1 that bind to PB2 and by enhancing vRNP export through binding of NP to LC3. The subsequent interaction between M2 and LC3 facilitated viral production by interfering with autophagosome maturation at later stages of infection. How LC3 binding switches from NP to M2 at different stages of infection remains to be elucidated.

Hemagglutinin (HA) and NS1 have also been documented to be involved in modulating the autophagy machinery. Similar to M2, HA alone can initiate autophagy, as proteolytic cleavage of highly pathogenic HA was shown to lead to accumulation of LC3-II protein [51]. Although NS1 alone is not sufficient to upregulate autophagy, NS1 has been known to facilitate viral replication by inhibiting apoptosis at early stages of infection. Therefore, it is speculated that NS1 first inhibits apoptosis and further promotes autophagosome formation by amplifying M2 and HA production [51]. Contrary to this hypothesis, Kuroki *et al.* [52] have proposed that NS1 inhibits autophagosome formation by suppressing c-Jun N-terminal kinase (JNK)-mediated autophagy and activating PI3K-AKT-mTOR pathway to prevent autophagic degradation of vRNP. It is worth noting that the extent of autophagy and apoptosis induction is cell line and virus-strain dependent [44,53–55]. Moreover, IAV is commonly known for inhibiting apoptosis at early stages of infection, to allow for sufficient time for viral replication and assembly, while triggering apoptosis at later stages of infection to facilitate propagation of infectious virions to neighboring uninfected cells [56]. Indeed, Yeganesh *et al.* [57] have demonstrated that IAV-induced apoptosis and viral release were greatly reduced when autophagy was inhibited

pharmacologically or was assessed in an autophagy-deficient cell line. The authors have also shown that, although pharmacological induction of autophagy seemed to enhance viral replication and protect cells from viral-induced apoptosis, the production of infectious viral particles was dramatically decreased [57]. These contradictory results can be explained if inhibiting autophagy resulted in limited availability of IAV proteins that normally trigger apoptosis [57]. On the other hand, Gannage *et al.* have shown that autophagy-deficient cells are more susceptible to apoptosis. M2 was also suggested to play an important role in modulating the extent of autophagosome-lysosome fusion, given that its deletion reduced the extent of apoptosis [44]. Autophagy and apoptosis are thus clearly interconnected pathways, and the delicate balance between them is very likely skewed by viruses to promote various steps in their life cycle (Fig. 2).

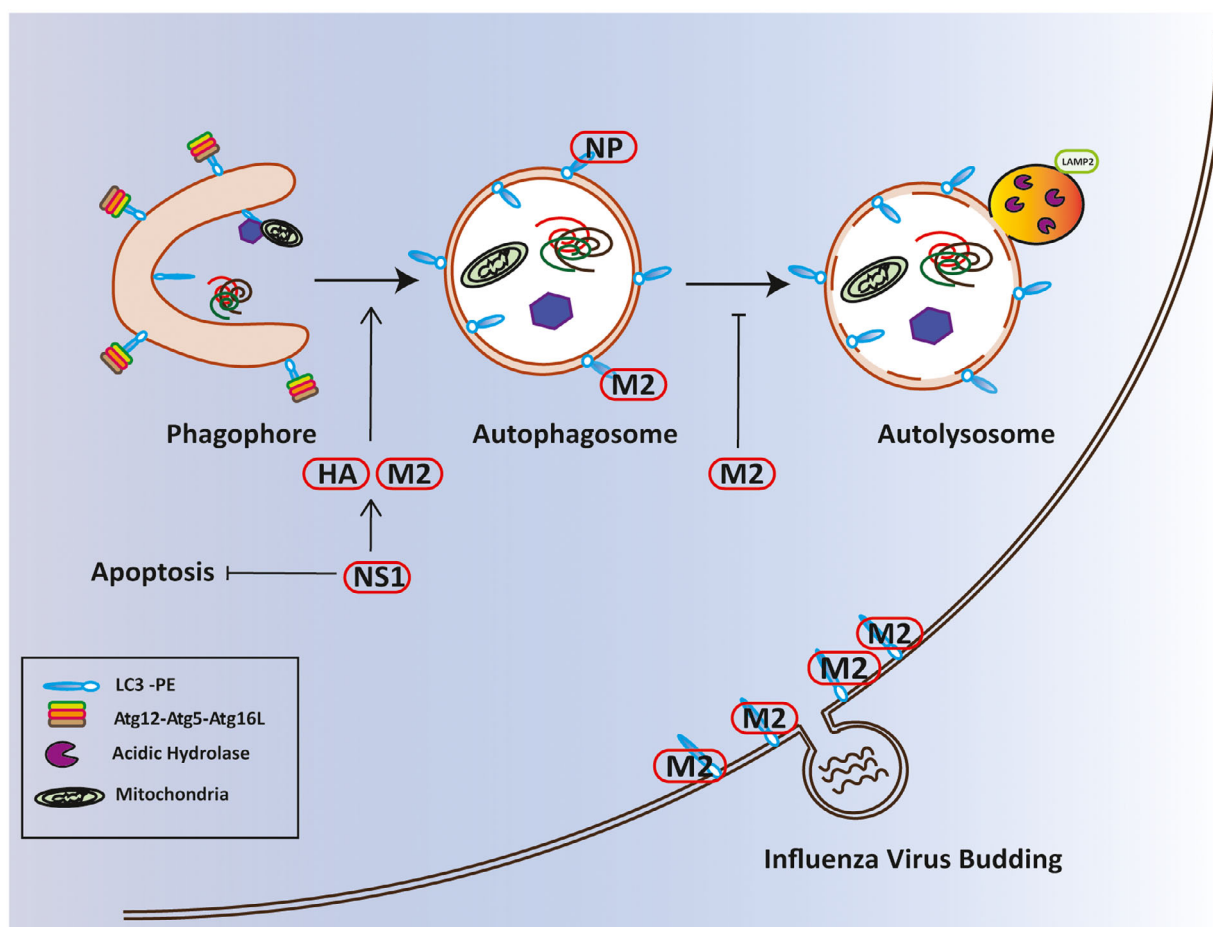
## Positive-strand RNA viruses

### Picornaviridae

(+) RNA viruses are more frequently associated with exploitation of autophagy, as this process allows for manipulation of intracellular membrane structures required for the establishment of the (+) RNA virus replication and assembly sites. The best characterized viral strategies for exploitation of secretory autophagy are described for positive-sense nonenveloped enteroviruses of the picornaviridae family. The pathway was first linked to these viruses, after nonlytic spread was observed by transmission electron microscopy (TEM) for these endogenously lytic viruses [58–60]. Multiple studies on poliovirus (PV), Coxsackie B3 virus (CVB3), and Enterovirus D86 (EV-D68) have shown that this nonlytic spread resulted from exploitation of secretory autophagy [58–62].

Most autophagy studies in enteroviruses have been conducted for Poliovirus 1 (PV). Autophagy was found to support poliovirus in viral particle maturation and subsequent release. The acidic environment of the amphisome was shown to be required to internally cleave the capsid protein VP0 and enable maturation of the provirion to become an infectious virus. In the absence of vesicle acidification, infectivity was significantly reduced [63]. It is currently not fully understood how these PV-containing amphisomes can be targeted for release at the cell surface, but it has been speculated that SNARE proteins are the main targets to redirect autophagy, as seen for other enteroviruses.

For example, for Enterovirus D68 (EV-D86) and Coxsackievirus B3 (CVB3), increased autophagosome



**Fig. 2.** Working model of Influenza viral subversion of autophagy for viral release. Influenza virus modulates autophagy at different stages of viral life cycle to enhance viral replication, assembly, and release. NS1 amplifies M2 and HA expression and inhibits apoptosis at early stages of infection. HA and M2 induce autophagy through an unknown mechanism. NP and M2 colocalize with LC3 predominantly at perinuclear regions and at the plasma membrane, respectively. NP and M2 induce autophagy to facilitate viral RNA synthesis and to enhance vRNP export. Additionally, M2 inhibits autophagosome–lysosome fusion and redirects LC3 to plasma membrane to facilitate viral release, possibly by interacting with Beclin-1 and LC3 through its highly conserved cytoplasmic tail.

formation was observed without lysosomal degradation [60–62]. It was suggested that degradation could be evaded by proteolytic cleavage of the SNARE protein SNAP29 of the SNARE autolysosome maturation complex (STX17-SNAP29-VAMP8 complex) and cleavage of the Pleckstrin homology and RUN domain-containing M1 (PLEKHM1) adaptor protein by these viruses, using their 3C protease (3C<sup>Pro</sup>). This cleavage at amino acid 161 on SNAP29 dissociates the C-terminal VAMP8-binding motif from the N-terminal STX17 interacting domain, thereby disrupting the SNARE autolysosome maturation complex and blocking autolysosomal fusion [62,64–66]. Moreover, the tethering complex of LC3-II, homotypic vacuole fusion and protein sorting (HOPS), and Rab7 cannot be

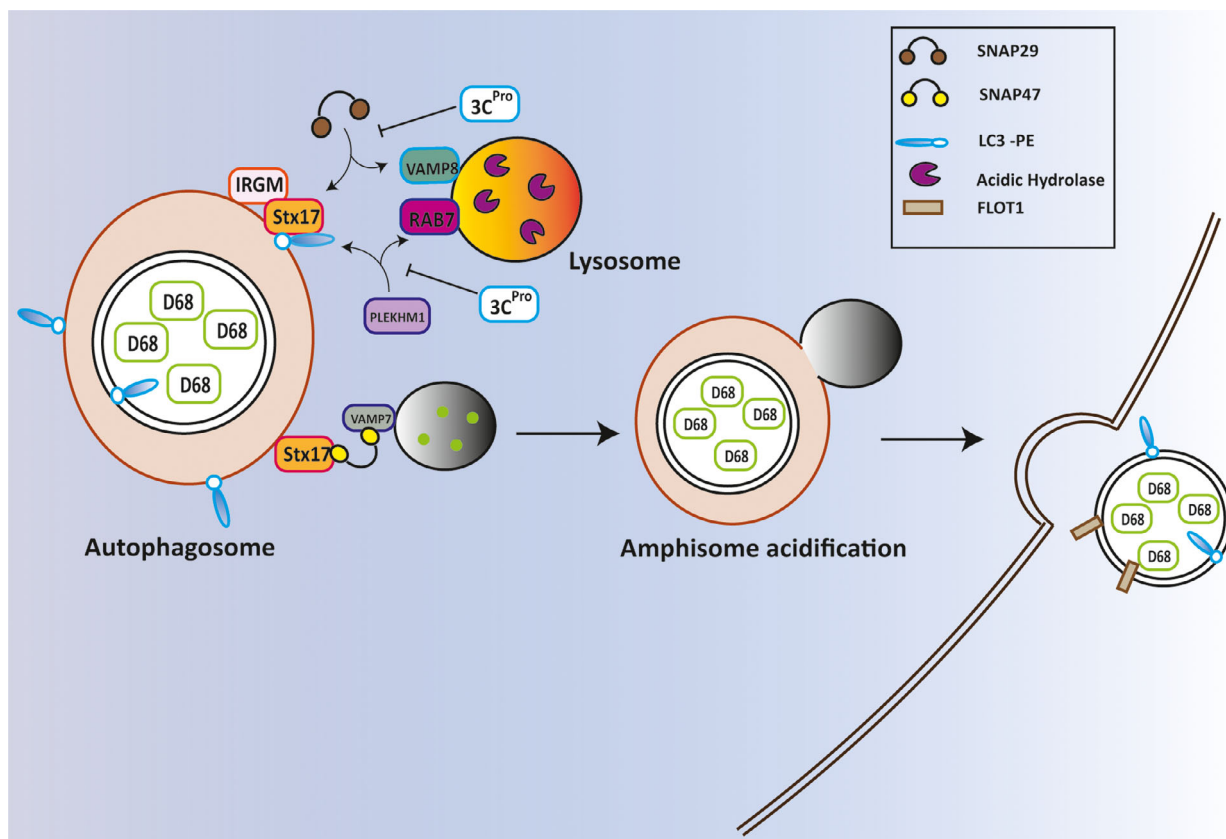
formed after cleavage of PLEKHM1, further preventing autolysosomal fusion [66]. EV-D68 3C<sup>Pro</sup> is a cysteine protease containing a Cys-His-Glu catalytic triad, and its three-dimensional structure is closely related to that of the 3C<sup>Pro</sup> of rhinovirus 2, as well as to that of poliovirus, both of which can employ a similar unconventional viral release mechanism [58,67]. By cleaving, SNAP29, CVB3 and EV-D86 inhibit lysosomal degradation, leading to accumulation of amphisomes in the cytosol. For EV-D68, cleaved SNAP29 was substituted by its homolog—the late endosome-associating SNARE—SNAP47, to allow for amphisome formation rather than autolysosome formation and support CVB3 maturation [62]. SNAP47 is a SNARE protein which can interact with the exocytic trafficking

molecule VAMP7, providing a possible link with viral release by an amphisome-dependent route [68]. However, this is a speculative model since experimental evidence to support that VAMP7 drives exocytic trafficking of these virus-containing amphisomes is currently lacking. Regardless of how these amphisomes can be trafficked to the cell surface for viral release, it was shown that these autophagic membranes were indeed used as the ‘envelopes’ for CVB3 release, as viral particles could be found in the extracellular space inside flotillin-1+, lipidated LC3-II+, unilamellar membranes, and resembling exosomes with autophagic markers [8,16,60] (Fig. 3).

### Flaviviridae

For many flaviviruses, including Zika virus (ZIKV) and Dengue virus (DENV), autophagy has also been suggested as a proviral factor. Flaviviral infections require

extensive ER reorganizations for efficient replication and assembly, which are thought to be predominantly supported by autophagy-mediated lipid droplet hydrolysis [69]. However, besides selective lipophagy, a general increase in autophagosome formation and modulation of autophagic flux can be observed for both flaviviruses, ZIKV, and DENV [70]. This increased autophagy induction was predominantly found to assist the virion maturation for ZIKV and DENV. Similar to Poliovirus, during initial infection only a moderate reduction in intracellular RNA synthesis, but a significant reduction of secreted infectious DENV particles, was detected upon inhibition of autophagy with the autophagy inhibitor Spautin-1, which destabilizes the Beclin-1-Vps34-ATG14 complex [71]. Similar to DENV, infection with ZIKV also corroborated this defect in assembly, maturation, and egress upon autophagy inhibition, with lowered RNA synthesis and infectious virus production in multiple rounds of infections [72].



**Fig. 3.** Working model of enteroviral exploitation of autophagy for viral release. EV-D68 skews degradative autophagy, to facilitate replication but predominantly virus maturation and release. 3C<sup>Pro</sup> of D68 cleaves SNAP29 of the SNARE autolysosome maturation complex (Stx17-SNAP29-VAMP8) preventing tethering of lysosomal and autophagosomal membranes for fusion. The endosome-localized SNAP47 can establish an alternative SNARE complex with Stx17 of the autophagosome and VAMP7, associated with exocytosis, to generate peripherally localized D68-containing amphisomes. The autophagosome–late endosome fusion is suggested to support Capsid maturation and the release of unilamellar LC3II+, Flotillin+, Phosphatidylserine-rich enterovirus-containing vesicles.



Specifically, the secretory pathway of autophagy could be linked to this role in DENV maturation, as inhibition of lysosomal fusion could increase virus titers. Furthermore, the autophagosomal marker LC3 was found to colocalize with dsRNA, DENV2 NS1, and mannose-6-phosphate receptor (M6PR) implying DENV2 virion localization in amphisomes [73]. These amphisomes were shown to not function as replication sites in two separate studies by Belov *et al.* [74] and by Mateo *et al.* [71], where the latter reported that impairment of autophagosome formation had minimal influence on DENV viral RNA replication. While amphisomes therefore do not appear to function as the dominant site of replication, impairment of these structures could hamper formation of infectious virus particles. Specifically, blocking autophagy prevented furin cleavage of the Pr peptide of PrM of DENV and thereby prevented maturation of the virus into infectious particles [71]. This suggests that these amphisomes predominantly assist in virus maturation. Amphisomes are formed as a result of autophagosome fusion (LC3II+) with late endosomes (M6PR+) and are classically acidified and targeted for degradation by fusion with a lysosome. As LC3II+ puncta were found to accumulate during DENV infection, the virus was hypothesized to inhibit lysosomal fusion and redirect acidified, LC3II+ vesicles to the cell membrane for release. Extracellular vesicles found in serum of a DENV-infected human and released from DENV-infected Huh7 cells were indeed detected to contain autophagic marker LC3-II, dsRNA, and viral proteins prM/M, NS1, and E, indicating that DENV-containing amphisomes are released [75]. Similarly in ZIKV, autophagosomes were also found to accumulate, allowing for increased viral replication and maturation. However, how these ZIKV and DENV-containing amphisomes escape lysosomal fusion and how they traffic to the cell membrane for release have not been completely elucidated. Recent studies have provided further insights on how amphisomes can be trafficked to the cell membrane for release during DENV infection [76]. The hypothesized amphisomal exit strategy of DENV was linked to the exploitation of the Src family kinases (SFKs). In particular, mature virions can utilize Lck/Yes novel tyrosine kinase (Lyn) of the SFK family to support anterograde transport of virion-containing amphisomes. It was shown that mature viruses could alternatively exit the cell with faster kinetics in specialized Rab11 and transferrin receptor-positive organelles resembling secretory autophagosomes in the presence of palmitoylated and catalytically active Lyn.

Besides amphisomal release, direct release of autophagosomes at the cell membrane was suggested

for a related flavivirus, Japanese encephalitis virus (JEV). During JEV infection, virus-associated vesicles carried nonlipidated LC3-I on their membranes rather than LC3II. Without lipidated LC3, autophagosomes are unable to fuse with lysosomes, but are suggested to still associate with membrane structures. As previously described for influenza A, a functional LC3-interacting region (LIR) motif was found on M2-protein that prevents this LC3 lipidation and thereby relocates LC3-I to the cell membrane to support viral budding [46]. This lipidation-independent secretory autophagy pathway has not been explored for flaviviruses, but LIR motifs have been identified for NS1, NS2a, and NS5 of DENV. These motifs should be explored on functionality, to test for similar mechanistic implications [72].

Unlike the previously mentioned flaviviruses, hepatitis C virus (HCV) does not induce autophagy but rather solely prevents degradation of autophagosomes by inhibiting autophagic flux early in infection. This virus is specifically known to usurp the autophagy pathway to establish chronic infection [77]. For HCV, autophagosomal fusion with lysosomes is inhibited by increasing the Arf-like GTPase Arl8b expression levels. Arl8b is an essential trafficking molecule involved in lysosomal positioning. Upon infection, the increased Arl8b expression levels result in lysosome repositioning to the periphery, to prevent fusion with autophagosomes [78]. As the rate of autophagosomal fusion is directly determined by the coordinated perinuclear localization of lysosomes and autophagosomes, repositioning of the lysosome could prevent fusion [79]. Rather than direct viral-induced fusion defects, trafficking molecules can thus be targeted for interfering with autophagy as well. Moreover, the temporal inhibition of autophagosomal fusion in HCV infection was hypothesized to also result from differential induction of the inhibitory Rubicon and stimulatory UVRAG protein of the SNARE autolysosome maturation support complex (HOPS-Beclin-1-UVRAG), stimulating autophagic flux late in infection [77]. The induction of increased UVRAG expression later in infection may also reflect altered endosomal trafficking to facilitate viral release, as UVRAG mediates enhanced endosomal maturation and transport [80,81].

The importance of elucidating the mechanism by which these flaviviruses can exploit autophagy for viral release was highlighted in a recent study by Zhang *et al.*, in which vertical transmission of ZIKV could be limited by inhibiting or gene silencing of Atg16L, a key component of the Atg5-Atg12 complex, and thereby could directly improve fetal and placental

outcomes [82]. Similarly for DENV, cell-to-cell spread was implicated to be promoted by secretory autophagy and suggested to directly contribute to evasion of neutralizing antibodies [75].

### Coronaviridae

Coronaviruses are no exception to the previously mentioned RNA viruses and numerous members of the Coronaviridae family, such as porcine epidemic diarrhea virus (PEDV) and Middle East respiratory syndrome coronavirus (MERS-CoV), are shown to have co-evolved to exploit autophagy for their own propagation [83–85]. Whether autophagy is required for viral replication, however, remains under contention and how these viruses could benefit from autophagy is still poorly understood. For the avian infectious bronchitis coronavirus (IBV), nonstructural protein 6 (nsp6) was found to induce autophagosome formation and block autophagic expansion during infection. Homologs for this nonstructural protein are encoded by severe and acute respiratory syndrome virus (SARS-CoV), mouse hepatitis virus (MHV), and the closely related arterivirus porcine reproductive and respiratory syndrome virus (PRRSV) [86].

A recent study implicated lysosomes in the unconventional egress of coronaviruses [87]. In line with previous studies, Arl8b, a small GTPase involved in autophagosome–lysosome fusion, regulated localization and exocytosis of lysosomes at the plasma membrane. Using MHV as a model system, the authors demonstrated that betacoronaviruses are able to prevent acidification of virus-containing lysosomes, inactivate lysosomal degradation, and instead target them for exocytosis using Arl8b [87].

While the mechanisms by which positive-sense RNA viruses are suggested to redirect autophagy from the degradative arm to the secretory arm are in no way completely resolved yet, Fig. 3 depicts the suggested secretory pathway for enterovirus EV-D68 as an example with the hypothesized viral and cellular components. Moreover, a table summarizing how these different RNA viruses have evolved similar strategies to intervene with degradative autophagy is shown in Table 1. Many questions remain on the role of autophagy in virus secretion and spread. Delineating viral exploitation mechanisms of autophagy from halted autolysosomal fusion for the benefit of egress of both negative and positive-sense RNA viruses may provide crucial insights into the molecular links between autophagy, endocytic pathways, and viral proteins.

### Conclusion

Autophagy is a well-known defense mechanism of the host, harnessed to degrade viral proteins, degrade complete viral particles, or even degrade host factors involved in the virus life cycle. However, in the evolutionary arms race between the host and these pathogens, many viruses have acquired strategies to subvert cellular autophagy in order to promote their own survival [59]. Exploiting the host secretory autophagy pathway for virus release can have significant implications for infectivity of a virus. First, release in these phospholipid-rich membranes could enhance viral uptake. Moreover, in the case of nonenveloped viruses, avoiding the highly inflammatory event of release by cell lysis, can reduce immune recognition and thus support viral survival [8]. Immune recognition can be further reduced, as neutralizing antibodies in the extracellular space cannot recognize viruses cloaked in these membranes. Finally, success of productive infection and evolution of fitter viruses can be further elevated as release in these 300–500 nm unilamellar vesicles provides these viruses the possibility to exit as quasispecies [88]. Furthermore, autophagy and apoptosis are two evolutionarily conserved processes that are closely linked together through several shared key regulatory proteins such as Beclin-1–Bcl2 interactions [89] and caspase-mediated Beclin-1 cleavage [90] to regulate cell fate. Viruses can therefore evolve to tip the balance between these cellular pathways at different stages of their life cycle for persistent viral infection and immune escape.

Despite major advances made in elucidating the complex interplay of RNA viruses with the host cellular autophagy pathway, several gaps remain in our understanding of viral exploitation of autophagy. First and foremost, the strategies by which viruses can redirect autophagosomes or amphisomes for release after blocking of autolysosomal fusion remain to be elucidated. The components involved in the equilibrium between autophagic vesicle degradation and secretion and the intersection with endosomal trafficking have to be further characterized, to understand the functional implications of viral interference in autophagy. Moreover, the strikingly big repertoire of RNA viruses that interfere with autophagy, many of which exploit different components and trigger noncanonical aspects of the pathway, highlights the complexity and redundancy present in cellular autophagy. The pathway must therefore be assessed in its entirety to understand how RNA viruses can co-opt it for both viral replication and

**Table 1.** RNA virus proteins associated with exploitation of autophagy for release and spread.

Autophagy components (autophagy steps)	Virus	Viral protein	Interaction of viral protein	Reference
Elongation & maturation: LC3	(–) RNA viruses Influenza A ( <i>Orthomyxoviridae</i> )	M2	At late stages in infection, M2 was found to interact with LC3 through the LIR motif to relocate to the cell membrane, preventing autolysosomal fusion, and inducing accumulation of autophagosomes. Viral envelope composition is potentially modulated by the remobilization of viral components in these LC3+ and M2+ autophagosomes	[33,86]
	HPIV-3 ( <i>Paramyxoviridae</i> )	M	Colocalization of LC3-I and Matrix protein (M) to enhance release at the cell membrane and to induce mitophagy to decrease IFN response	[27].
	SNV, HNTV ( <i>Bunyaviridae</i> )	Gn	Specific colocalization of Gn with LC3-II to selectively degrade the glycoprotein to allow for efficient replication, besides attenuated IFN response, via mitophagy for potential persistence	[37,38]
	(+) RNA viruses DENV, ZIKV ( <i>Flaviviridae</i> )	NS1, dsRNA	Colocalization with amphisomes indicates their contribution to viral replication as alternative replication sites; required for efficient PrM cleavage	[71,73]
	JEV/DENV ( <i>Flaviviridae</i> )	NS1, NS2a, NS5	DENV proteins containing LIR motifs, hypothesized to support lipidation-independent secretory autophagy as seen for JEV	[46,71,72]
	(–) RNA viruses Influenza A ( <i>Orthomyxoviridae</i> )	PB1-F2, NS2	IAV proteins found to associate with IRGM	[33].
Maturation: IRGM	MeV ( <i>Paramyxoviridae</i> )	C	Support viral life cycle by induction of efficient autophagy by recruitment of LC3, Atg5, Atg10 using IRGM and delaying apoptosis to allow for cell-to-cell spread	[30,32,33]
	(–) RNA viruses HPIV-3 ( <i>Paramyxoviridae</i> )	P	Phosphoprotein P binds SNAP29 hindering interaction with Stx17 and VAMP8	[26]
Maturation: STX17-SNAP29-VAMP8 complex	HNTV ( <i>Bunyaviridae</i> )	NP	Counteracts Gn autophagic degradation by competitive binding to Stx17, thereby preventing autolysosomal fusion during the second wave of autophagy	[37]
	(+) RNA viruses EV-D68, CVB3 ( <i>Picornaviridae</i> )	3C <sup>Pro</sup>	Proteolytic cleavage of SNAP29 by this viral protein results in autolysosomal fusion arrest	[62,64–66]
	(+) RNA viruses EV-D68, CVB3 ( <i>Picornaviridae</i> )	3C <sup>Pro</sup>	Proteolytic cleavage of PLEKHM1 by this viral protein results in autolysosomal fusion arrest	[62,64–66]
Maturation: PLEKHM1-LC3-Rab7-HOPS tethering complex	HCV ( <i>Flaviviridae</i> )	?	Differential induction of Rubicon and UVRAG by an unknown viral mechanism leads to block in autolysosome fusion early in infection and stimulation later in infection to facilitate viral release. Moreover increased Arl8b expression leads to repositioning of Rab7+ lysosomes preventing autolysosomal fusion	[77,78,80,81]
	(–) RNA viruses Influenza A ( <i>Orthomyxoviridae</i> )	NS2, M2	IAV proteins found to associate with UVRAG. M2 was found to bind Beclin-1 and thereby prevent autolysosomal fusion and accumulation of autophagosomes	[33,44]

spread, while preventing virus degradation and immune sensing, in order to identify potential universal drug targets while avoiding viral benefits of autophagy.

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## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

QWT, SWvL, and SS discussed and wrote the manuscript. SS supervised the study.

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