CHAPTER VI

LC3 Proteins Play a Crucial Role in Influenza Virus Infection

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Abstract

Influenza A virus (IAV) is a membrane-enveloped virus with a segmented, single strand negative-sense RNA genome, which causes annual epidemics and occasional pandemics. Each of the eight genomic segments forms a ribonucleoprotein (vRNP) complex together with multiple copies of viral nucleoprotein and a single copy of the viral RNA polymerase complex. The role of vRNPs in IAV life cycle is to orchestrate viral RNA replication and transcription, intracellular viral RNA transport, gene re-assortment as well as genome packing into progeny particles. For the successful completion of virus life cycle, vRNPs also rely on host cellular processes to perform the necessary functions. Autophagy is a conserved catabolic process for the recycling of cytoplasmic macromolecules and is also critical for the interaction between IAV and the host. However, the function of ATG proteins during IAV infection remains to be fully understood. Here, we confirm that IAV triggers an autophagic response in host cells but we also show that an intact ATG machinery is not required for the IAV life cycle. Nevertheless, depletion of LC3 members of ATG8 protein family crucially impairs IAV infection revealing their pivotal role in IAV intracellular life. Moreover, LC3 proteins but not GABARAP proteins, associated with part of the cytoplasmic vRNPs in their non-lipidated forms. Our data here reveal an autophagy-independent role of LC3 proteins in IAV infected host cells suggesting the differential requirement of autophagy components during the viral life cycle.

Keywords: Influenza A virus, autophagy, LC3, vRNP
1. Introduction

Influenza viruses are enveloped viruses with a segmented genome consisting of single-stranded, negative-sense RNA and belong to the *Orthomyxoviridae* family, which is sub-classified in type A, B, C and the recently identified D (1, 2). Since influenza A virus (IAV) causes millions of deaths in pandemic years, most of the studies focus on this influenza type and not the other ones (3, 4). The IAV genome is composed of 8 RNA segments that encode transcripts for 11 proteins (5, 6). Two of them, NS1 and NS2, are non-structural proteins; of these NS1 is well-characterized, as it is essential for virus pathogenicity (7-9). The rest (PB2, PB1, PA, HA, NP, NA, M1 and M2) are structural proteins and compose the viral particles. NP is one of the most abundantly expressed viral proteins and is the main component of viral ribonucleoproteins (vRNPs), which are key for the encapsulation of the viral genome (9). The viral RNA genomic segments are wrapped around an oligomeric filament composed of NP that is bound at one of its extremities to a single copy of heterotrimeric viral polymerase, which consists of PB1, PB2 and PA (5).

IAV enters the host cell via receptor-mediated endocytosis and after fusion of the viral and endosomal membranes, the vRNPs present in the interior of virion are released into cytoplasm and then transported into nucleus, where the transcription occurs. The newly translated NP and PA, PB1, PB2 relocate to nucleus to form vRNPs while M1, HA, NA and M2 concentrate at plasma membrane. The newly assembled vRNPs are exported from nucleus into cytoplasm before being directed to plasma membrane where the interaction with M1 allows their incorporation into forming viral particles (10). The NP protein principally localizes to nucleus at the early stage of infection, but later forms distinct punctate structures, which represent vRNPs, exported from nucleus to cytoplasm (11, 12). Not surprisingly, several reports have shown that the host nuclear pore components such as karyopherin subunits α1 (KPNA1/NPI-1/SRP-1) and α2 (KPNA2/NPI2/RCH1), IMPORTIN5 (IPO5/KPNA3), CRM1 and nuclear transport factor 2-like export protein 1 (NXT1), assist vRNPs nuclear transport by interacting with NP (13-17). In addition, NP also binds to other cellular factors to promote viral replication and suppress host innate immune response, including the DExD-box helicase 39B (DDX39B/BAT1/NPI5/UAP56), high mobility group box 1 (HMGB1), CCR4-NOT transcription complex subunit (CNOT4) and heat shock protein 40 (HSP40) (18-21). It still remains unknown, whether the assembly of larger vRNPs in
cytoplasm and their subsequent transport to plasma membrane to be incorporated in the forming virions needs other cellular factors.

Macroautophagy (hereafter autophagy) is a catabolic pathway essential for maintaining cellular homeostasis and is also involved in numerous cellular and organismal functions (22-24). Cytoplasmic structures targeted by autophagy are sequestered into double-membrane vesicles called autophagosomes and delivered into lysosomes for degradation (22, 25). The formation of autophagosomes is orchestrated by a core set of conserved proteins termed autophagy-related (ATG) genes (26). In particular, autophagosome biogenesis is initiated by the ULK complex, transmembrane ATG9A and the class III phosphatidylinositol 3-kinase complex, which generates phosphatidylinositol-3-phosphate (PtdIns3P) on the precursor membrane known as phagophore (27, 28). Subsequently, interactions of this lipid and/or specific proteins lead to the recruitment of the rest of the ATG machinery that mediate the expansion and closure of the phagophore (29). This includes two ubiquitin-like proteins, ATG12 and ATG8. ATG12 is activated by ATG7, an E1-like enzyme, and then transferred to ATG10, an E2-like enzyme, before being conjugated to ATG5 (24, 30). The ATG12-ATG5 conjugate then forms a supramolecular complex with ATG16L1. Eukaryotes have from one to several ATG8 proteins. The human genome encodes for six ATG8 proteins that are divided into LC3 and GABARAP protein subfamilies, which include LC3A, LC3B, LC3C, and GABARAP, GABARAPL1 and GABARAPL2, respectively (31, 32). ATG8 proteins are post-translationally processed by the ATG4 cysteine proteases to expose a C-terminal glycine residue (33). Upon autophagy induction, ATG7 activates the ATG8 proteins and ATG3, an E2-like enzyme, in concert with the ATG12-ATG5-ATG16L1 complex, subsequently mediates their conjugation to phosphatidylethanolamine (PE) (34). The ATG12-ATG5-ATG16L1 complex determines the membrane where ATG8 proteins get linked to PE and over the course of autophagy, it leads to the formation of ATG8-PE on both the inner and outer surface of nascent autophagosomes. The different ATG8-PE proteins on autophagosomes have distinct functions that have been associated with closure, fusion with lysosomes, degradation of the internal lipid bilayer and autophagosome transport (32). Moreover, ATG8 proteins function as adaptor proteins to recruit selective cargo to the autophagosome via interaction with cargo receptors during selective types of autophagy (35). Thus, ATG8 proteins have been widely used to monitor the autophagic activity as well as the number of autophagosomes (35). ATG8-PE can also be generated on single membrane structures like endosomes and plasma membrane, where they can promote
phagosome maturation, signaling and fusion (36). Specific cellular functions have also been linked to non-lipidated forms of ATG8 proteins. LC3 is involved in a trafficking route out of the ER known as the endoplasmic reticulum (ER)-associated degradation (ERAD) tuning pathway (37, 38), which appears to be hijacked by the mouse hepatitis virus (MHV), the equine arteritis virus (EAV) and the Japanese encephalitis virus (JEV) for generation of the intracellular membrane rearrangements where those viruses mainly replicate (39-41). Similarly, non-lipidated LC3 associates with *Chlamydia trachomatis* inclusions and it is essential for the intracellular growth of this bacterium (42).

Autophagy is crucial for cellular defense against invading pathogens by restricting the growth of some of them in cytosol (43). In addition, it participates to multiple other aspects of our adaptive immunity, including delivering cytoplasmic antigens to the MHC class II loading compartment or regulating T-cell homeostasis (44). As a result, numerous pathogens have developed mechanisms to subvert autophagy by either inhibiting this pathway or hijacking part of ATG machinery, to promote their propagation in the host (45-49). IAV also appears to modulate autophagy but the molecular details and the relevance of this interaction remain largely elusive. An increasing number of studies has shown using electron microscopy, GFP-LC3 staining and/or the biochemical analysis of LC3 lipidation, that IAV infection induces autophagy in a variety of cell lines (50-55). The inhibition of class III phosphatidylinositol 3-kinase complex with unspecific compounds such as 3-methyladenine (3-MA) or wortmannin, or knockdown or knockout of specific ATG proteins, including ATG3, ATG5, ATG7, BECLIN1 and LC3B, lead to a reduction of IAV replication, indicating that autophagy, or a subset of its components, plays an important role in the life cycle of this virus (50, 55-61). Conversely, induction of autophagy through rapamycin, a drug that changes the cell metabolism, enhances IAV replication (50, 56, 62). Although some of these studies indicate that autophagy is crucial in the early phases of IAV infection, other reached the conclusion that it is more important at a later stage. In this regard, it has been shown that although autophagosome formation is triggered by IAV infection, these vesicles do not fuse with lysosomes and therefore the autophagic flux is not required to sustain the replication of this virus (52). Moreover, GFP-LC3B initially concentrates in perinuclear puncta representing autophagosomes when cells are exposed to IAV, but redistribute to plasma membrane in the late time points of the infection (52, 53, 63). M2 is emerging as a critical protein in autophagy regulation over the course of IAV infection and it is sufficient to induce autophagosome formation (50, 52, 64). M2 also possesses a LC3-
interacting region (LIR) motif that directs transfected GFP-LC3B to plasma membrane (53). This interaction between M2 and LC3 is pivotal for filamentous budding and its ablation reduces progeny virion stability (53). Different studies have reported, however, a different distribution pattern between endogenous LC3 compared to GFP-LC3, which also forms punctate structures but they are not redistributed distinctively to plasma membrane upon IAV infection even at late stage (55, 56, 65).

With our investigation, we aimed at understanding better the interaction between IAV and the ATG machinery, in particular ATG8 proteins. We found that an intact ATG machinery is not required for IAV infection. A subset of non-lipidated ATG8 proteins, however, associates with part of the cytoplasmic vRNPs. In particular, members of the LC3 protein subfamily but not those belonging the GABARAP protein subfamily, play a crucial role in IAV infection. Thus, our data reveal that although an intact autophagy pathway is not required for IAV infection, non-lipidated LC3 proteins play an essential role in the life cycle of this virus.

2. Results

2.1 Endogenous LC3 co-localizes with IAV NP protein

Induction of autophagy upon IAV infection has been observed in a number of cell lines by evaluating both lipidation of LC3 proteins and the formation of GFP-LC3-positive autophagosomes (52, 53, 59). To understand better the interaction between autophagy and IAV, we first monitored induction of autophagy over the course of IAV infection in U2OS cells by assessing the steady-state levels of LC3-I and LC3-II. A significant induction of LC3 conversion was observed at 16 and 24 hours post infection (hpi), when substantial levels of viral NP and M2 were also detectable (Fig. 1A and 1B). This result confirms previous data showing that IAV infection triggers autophagy.

To specifically examine autophagy induction in the subpopulation of infected cells, we repeated the same experiment but analyzed the samples by fluorescence microscopy. NP staining was used to identify infected cells. In agreement with the western blot analysis, the number of endogenous LC3-positive puncta increased over the course of the infection and it was particularly prominent at 16 and 24 hpi (Fig. 1C and 1D). Very surprisingly, numerous cytoplasmic NP-positive vRNPs co-localized with endogenous LC3 (Fig. 1C and 1E).
**Figure 1. Endogenous LC3 co-localize with IAV NP protein in U2OS cells.** U2OS cells were infected with IAV at a multiplicity of infection (moi) of 0.1 before being processed for western blot and IF at 0, 8, 16 or 24 hpi. A. Cell extracts were separated by SDS-PAGE and western blot membranes probed with antibodies against NP, M2, LC3 and β-actin (loading control). B. Quantification of NP/actin, M2/actin and LC3-II/actin ratios in the experiment shown in panel A. Error bars represent the SD of 3 independent experiments. Asterisks indicate significant differences (p <0.01). C. Cells processed for IF were stained with anti-NP and anti-LC3 antibodies. Size bar, 5 μm. D. Quantification of LC3 puncta per cell in the experiment shown in panel C. Error bars represent the SD of 3 independent experiments. Asterisks indicate significant differences (p <0.01). E. Quantification of the NP puncta positive for LC3 per cell in the experiment shown in panel C. Error bars represent the SD of 3 independent experiments. Asterisks indicate significant differences (p <0.01).

This analysis was repeated in A549 cell line, which is frequently employed for studies on IAV, to exclude that this peculiar phenotype was cell line-specific. Consistent with the observations done in U2OS cells, we found that IAV was inducing autophagy in A549 cells over time and that part of cytoplasmic vRNPs were positive for endogenous LC3 (Fig. 2).

**2.2 GFP-LC3 chimera is not recruited to vRNPs**

It has been previously reported that in IAV-infected cells ectopically expressed GFP-LC3 initially accumulates in autophagosomes that concentrate in the perinuclear region, and subsequently partially re-distributes to plasma membrane at later stages of the infection through interaction with M2 (52-54). As we could not detect endogenous LC3 at plasma membrane and different distribution patterns between endogenous LC3 and GFP-LC3 have been observed in cells infected with coronavirus (39), we analyzed the localization of both the endogenous and the ectopically overexpressed LC3 in cells exposed to IAV.

The subcellular distribution of endogenous LC3 and GFP-LC3B was examined in A549 cells at 16 hpi. Endogenous LC3 was distributed in numerous puncta and several overlapped with the vRNPs signal (Fig. 3A and 3B). Those puncta, however, were not co-localizing with intracellular M2, which is initially synthesized and inserted into ER membranes (73). In contrast and in agreement with the literature (52, 53, 59), part of GFP-LC3B accumulated in puncta in the perinuclear region of infected cells, very likely autophagosomes, while part was observed together with M2 at plasma membrane at the same post-infection time point. Surprisingly GFP-LC3B did not co-localize to vRNPs (Fig. 3B). These data were also confirmed in U2OS cells (Fig. S1).

Altogether, these data show that endogenous LC3, but not the bona fide autophagosomal protein marker GFP-LC3, associates with cytoplasmic vRNPs.
Figure 2. Endogenous LC3 co-localize with IAV NP protein in A549 cells. A549 cells were infected with IAV at a moi of 0.1 before being processed for western blot and IF at 0, 8, 16 or 24 hpi. A. Cell extracts were separated by SDS-PAGE and western blot membranes probed with antibodies against NP, M2, LC3 and β-actin (loading control). B. Quantification of NP/actin, M2/actin and LC3-II/actin ratios in the experiment shown in panel A. Error bars represent the SD of 3 independent experiments. Asterisks indicate significant differences (p <0.01). C. Cells processed for IF were stained with anti-NP and anti-LC3 antibodies. Size bar, 5 μm. D. Quantification of LC3 puncta per cell in the experiment shown in panel C. Error bars represent the SD of 3 independent experiments. Asterisks indicate significant differences (p <0.01). E. Quantification of the NP puncta positive for LC3 per cell in the experiment shown in panel C. Error bars represent the SD of 3 independent experiments. Asterisks indicate significant differences (p <0.01).

2.3 Non-lipidated LC3 associates with vRNPs

Next, we investigated whether autophagy is required for IAV life cycle by analyzing the infection of this virus in cells lacking ATG7 or ATG13 (Fig. S2A), two genes essential for autophagy (74). The atg7−/− and atg13−/− knockout cells and their parental U2OS cell line were infected with IAV and analyzed by IF at 8 and 12 hpi to determine the percentage of infected cells. No significant differences were detected between the parental and knockout cell lines at the analyzed time points (Fig. 4A). We also assessed the levels of NP in the same cell line at 16 or 24 hpi to substantiate this observation. This experiment confirmed that deletion of ATG7 or ATG13 does not negatively or positively affect IAV infection as no significant differences in the cellular amounts of these viral proteins were detected at these hpi time points (Fig. 4B and 4C). Altogether, these results show that an intact host autophagy machinery is dispensable for IAV infection.

The conjugation of ATG8 proteins to PE is an essential step during autophagy and involves several ATG proteins, including ATG7 (75) (Fig. S2A). The observation that IAV infection is not altered in the absence of ATG7 (Fig. 4A-4C), promoted us to examine whether LC3 was still associated to vRNPs in IAV-infected atg7−/− cells. Very interestingly, LC3 association to NP puncta was not affected by the depletion of ATG7, revealing that the non-lipidated form of LC3 is recruited to the vRNPs (Fig. 4D and 4E). The same analysis was also performed in atg13−/− cells, which present a partial defect in LC3 conjugation (Fig. S2A), and LC3 association to vRNPs was unaltered in this cell line as well (Fig. S2B and S2C).

Collectively, these data show that the intact host autophagy machinery is dispensable for IAV infection as well as for the association of non-lipidated LC3 to vRNPs.
Chapter VI

A

LC3  NP  DAPI  merged  inset

LC3  M2  DAPI  merged  inset

GFP-LC3  NP  DAPI  merged  inset

GFP-LC3  M2  DAPI  merged  inset

B

![Graph showing LC3-positive NP puncta percentage]
LC3 Proteins Play a Crucial Role in IAV infection

2.4 IAV infection requires a specific subset of ATG8 proteins

The mammalian ATG8 protein family consists of six members, which are subdivided into LC3 and GABARAP protein subfamilies (76). To determine whether the members of the LC3 or GABARAP protein subfamily play an important role in IAV life cycle, we took advantage of two cell lines, LC3 TKO and GABARAP TKO, which lack the three members of LC3 and GABARAP protein subfamily, respectively (67) (Fig. S3A-S3C). LC3 TKO and GABARAP TKO, and their parental sHeLa cell line were infected with IAV for 12 h before being processed for IF (Fig. 5A). The number of infected cells was pronouncedly and significantly reduced in LC3 TKO, compared to parental and GABARAP TKO cells. Analysis of viral NP protein production by western blot over the course of IAV infection further validated this finding (Fig. 5B and 5C).

We also corroborated this observation by exploring whether GABARAP associates to cytoplasmic vRNPs during an IAV infection. Therefore, we simultaneously stained sHela and A549 cells for GABARAP and NP at 16 hpi (Fig. 5D and S3D). In contrast to LC3, GABARAP proteins did not significantly co-localize to vRNPs (Fig. 5D and 5E). This observation further supports the finding that GABARAP proteins do not play a crucial role in IAV infection.

Next, we back-transfected LC3 TKO cells with plasmids expressing the different LC3 protein subfamily members individually or in combination, to demonstrate whether the observed impairment in IAV life cycle was indeed due to the lack of one or more of these proteins. Expression of LC3A, LC3B, LC3C or of the 3 proteins at the same time, restored IAV infection in LC3 TKO cells, which was measured by monitoring NP protein synthesis at 16 hpi (Fig. 6A and 6B).

We also tested the outcome of LC3 protein knockdown on IAV infection in a different cell line using siRNA interference, to exclude a cell type-specific phenotype. We opted for atg7−/− U2OS cells to exclude that the observed effects on the virus life cycle could be due to an alteration of autophagy or any other pathway unconventionally involving the lipidated LC3 proteins. The siRNA probes led to a substantial but also complete depletion of LC3 (Fig. 6C).
Figure 4. Non-lipidated LC3 associates with IAV NP protein. A. Wild type (WT), atg7−/− and atg13−/− U2OS cells were infected with IAV at a moi of 0.1 before being processed for IF at 8 or 12 hpi. Cells were stained with anti-NP antibodies to determine the percentage of NP-positive infected cells. Error bars represent the SD of 3 independent experiments. B. WT, atg7−/− and atg13−/− U2OS cells were infected with IAV at a moi of 0.1 for 0, 16 and 24 h. Cell extracts were then separated by SDS-PAGE and western blot membranes probed with antibodies against NP and β-actin (loading control). C. Quantification of NP/actin ratios in the experiment shown in panel B. Error bars represent the SD of 3 independent experiments. No significant difference were observed between the WT and the knockout at each hpi. D. WT and atg7−/− U2OS cells were infected with IAV at a moi of 0.1 before being processed for IF at 12 hpi and stained with anti-NP and anti-LC3 antibodies. Size bar, 5 μm. E. Quantification of the NP puncta positive for LC3 in the experiment shown in panel D. Error bars represent the SD of 3 independent experiments.

The pronounced reduction of the cellular levels of LC3 proteins caused a significantly decreased in NP synthesis in IAV-infected atg7−/− knockout compared to cells treated with scramble siRNA, at both 16 and 24 hpi (Fig. 6C and 6D).

In sum, our data show that the members of LC3 protein subfamily are recruited to vRNPs and play a crucial role in IAV infection though an autophagy independent mechanism.

3. Discussion

Although the autophagy pathway is recognized as a key component of the host defense, growing evidence has revealed that the ATG machinery is subverted by specific viruses in order to promote their replication and spread, for example EMCV and HCV (77-79). This has prompted a number of groups to investigate the role of autophagy in IAV infection and eventually how IAV modulate this pathway. This topic, however, is still the subject of an ongoing debate. Although numerous studies have shown that autophagy is induced over the course of an IAV infection, it remains to be firmly established whether this pathway is essential for the life cycle of IAV (50, 52, 56, 58). Our observations are consistent with the notion that IAV infection triggers autophagy as we detected the formation of numerous LC3 or GFP-LC3 puncta upon exposure of different cell lines to this virus (Fig. 1 and 2). Our results obtained in atg7−/− or atg13−/− cells, however, show that an intact autophagy pathway is not essential for IAV infection at least not in our experimental setup (Fig. 4A-4C).

We found differences in the subcellular distribution between endogenous LC3 and ectopically expressed GFP-LC3B. In agreement with the literature, both endogenous LC3 and GFP-LC3 forms punctate structures upon IAV infection (Fig. 1C, 1D, 2C and 2D) (55, 56, 65), but part of those positive for endogenous LC3 proteins are cytoplasmic vRNPs (Fig. 1C, 1D, 2C and 2D). In contrast,
LC3 Proteins Play a Crucial Role in IAV infection

**Figure 5. LC3 proteins play a key role in IAV infection.** A. sHela, LC3 TKO and GABA TKO cells were infected with IAV at n moi of 0.1 before being processed for IF at 12 hpi. Cells were stained with anti-NP antibodies to determine the percentage of NP-positive infected cells. Error bars represent the SD of 3 independent experiments. Asterisks indicate significant differences (p <0.01). B. sHela, LC3 TKO and GABA TKO cells were infected with IAV at a moi of 0.1 for 0, 16 and 24 h. Cell extracts were separated by SDS-PAGE and western blot membranes probed with antibodies against NP and β-actin (loading control). C. Quantification of NP/actin ratios in the experiment shown in panel B. Error bars represent the SD of 3 independent experiments. Asterisks indicate significant differences (p <0.01). D. sHela cells were infected with IAV at a moi of 0.1 before being processed for IF at 16 hpi. The cells were stained with anti-NP and anti-LC3 or anti-GABARAP antibodies. Size bar, 5 μm. E. Quantification of the NP puncta positive for LC3 or GABARAP in the experiment shown in panel D. Error bars represent the SD of 3 independent experiments. Asterisks indicate significant differences (p <0.01).

GFP-LC3B does not associate with vRNPs. This chimera, however, was detected at plasma membrane during the late stage of IAV infection (Fig. 3), which is consistent with previous reports (52, 53). In apparent discrepancy with the previous data, we have not observed co-localization between endogenous LC3 and M2. One simple explanation could be that the cellular levels of endogenous LC3 are less than those of ectopically overexpressed GFP-LC3B and therefore it is more difficult to be detected at the plasma membrane. Another option could be that the LC3 antibody used in our study can only detect LC3A and LC3B (Fig. 6A), and one cannot exclude that endogenous LC3C is the major isoform of LC3 proteins that is targeted to plasma membrane by M2.

Consistent with the notion that LC3 has the autophagy-independent role during IAV infection, we observed that endogenous LC3 co-localized with vRNPs even in the absence of ATG7 (Fig. 4D and 4E), which reveals that conjugation to PE is not required for LC3 association to these complexes. Interestingly, non-lipidated LC3 proteins have also been found to be associated with the membranous replication platforms of MHV, EAV and JEV, which appear to be derived from the ERAD tuning pathway (39-41). Depletion of LC3A and LC3B was sufficient to block the replication of these viruses. When we targeted LC3A and LC3B individually or in combination by siRNA, no significant change in IAV infection progression was detected (data not shown), consistent with our observation that IAV infection is strongly impaired in LC3 TKO and each of the LC3 proteins is autonomously able to complement this defect (Fig. 5 and 6). These results suggest that the role of LC3 in IAV replication might be different from the one in the life cycle of the above-mentioned viruses, where LC3 appears to be subverted to divert membranes.
LC3 Proteins Play a Crucial Role in IAV infection

Figure 6. Non-lipidated forms of LC3s are essential for IAV infection. A. LC3 TKO cells were transfected with empty vector or a plasmids expressing LC3A, LC3B, LC3C individually or in combination (LC3s) for 48 h before to be infected with IAV at an moi of 0.1. Cells were collected at 16 hpi and cell extracts separated by SDS-PAGE and western blot membranes probed with antibodies against NP, LC3 and β-actin (loading control). B. Quantification of NP/actin ratios in the experiment shown in panel A. Error bars represent the SD of 3 independent experiments. Asterisks indicate significant differences (p <0.01). C. The atg7−/− U2OS cells were transfected with scramble siRNA or a pool of siRNA targeting the 3 members of the LC3 protein subfamily (siLC3s) for 48h and then infected with IAV at an moi of 0.1. Cells were collected at 0, 8, 16 and 24 hpi and cell extracts separated by SDS-PAGE before to probe membranes by western blot with antibodies against NP, LC3 and β-actin (loading control). D. Quantification of NP/actin ratios in the experiment shown in panel C. Error bars represent the SD of 3 independent experiments. Asterisks indicate significant differences (p <0.01).

In our study, we show that LC3 plays an important role in IAV infection and systematically investigated the relevance of each member of Atg8 protein family in IAV life cycle. IAV infection in LC3 TKO and GABARAP TKO cell lines revealed that only the members of the LC3 protein family are of crucial relevance (Fig. 5). Our data are in agreement with studies where it was revealed that silencing of LC3B hampers IAV infection (50, 55) and it was suggested to be due to the role of autophagy in stimulating ROS production, which promotes IAV life cycle at the early steps of the infection (55). The complementation of IAV infection defect in the LC3 TKO by back-transfection of LC3 proteins individually or in combination, showed that they have redundant function in IAV life cycle (Fig. 6A and 6B). To further underline that the non-lipidated form of these proteins contributes to IAV infection, we knocked down all 3 isoforms in atg7−/− cells and this led to an impairment of IAV infection, confirming that LC3 proteins are crucial for IAV infection outside the context of autophagy (Fig. 6C and 6D).

LC3 is abundant in nucleus despite the fact that it functions primarily in the cytoplasm where autophagosomes arise (80). In nutrient-rich conditions, LC3 is distributed in an acetylated form in both nucleus and cytoplasm, and nutrient deprivation leads to the redistribution of LC3 from the nucleus to the cytoplasm. This relocation depends on LC3 deacetylation by the activated nuclear deacetylase SIRT1 and its binding partner tumor protein p53 inducible nuclear protein 2 (TP53INP2/DOR) (80). TP53INP2/DOR, together with the nuclear protein HMGB1, are translocated to the cytoplasm upon starvation and participate in regulation of autophagosome biogenesis and protein degradation (81, 82). Interestingly, HMGB1 also binds to NP to promote viral replication in the nucleus at the early stage of infection, which suggests that the association between vRNP and LC3 could already occur at this step, but this speculative hypothesis still needs to be further investigated.
What is the function of LC3 protein when associated with the vRNPs? Individual vRNPs assemble in the nucleus and undergo nuclear export after M1-mediated release from the nuclear matrix (83, 84). The vRNP-M1 complexes are thought to be associated with NS2/NEP, which allows an interaction with the cellular CRM1 nuclear export machinery and promotes vRNPs transport into the cytoplasm through nuclear pore complexes in a NS2/NEP-dependent manner (85). Following nuclear export, vRNPs initially accumulate near the microtubule-organizing center and subsequently may associate with microtubules during rerouting to plasma membrane (86). LC3 proteins belong to the protein family of microtubule-associated proteins (MAP) and interact with MAP1A or MAP1B to form a complex that binds and modulates the shape of microtubules (87, 88). Thus, it may be possible that LC3 proteins assist the organization and/or transport of vRNPs from near the microtubule-organizing center to the plasma membrane for viral particle assembly. Interestingly, cytoplasmic non-lipidated LC3 interacts with the *Chlamydia trachomatis* inclusions as a microtubule-associated protein rather than an autophagosomal component (42). Similar to what we observed during IAV infection, GABARAP proteins appear not to play a role in *Chlamydia* infection (42). Other possible functions of LC3 proteins in IAV life cycle, however, cannot be excluded a priori.

Future studies are needed to unveil the function of LC3 proteins at the IAV vRNPs. Increasing our understanding of this relationship will greatly improve our knowledge of the pathogenesis of IAV but also provide an insight into the design of candidate antiviral therapeutics for more specific targeting of this life-threatening pathogen.

4. **Materials and Methods**

4.1 **Cell culture and virus**

U2OS, A549, sHela and Madin-Darby canine kidney (MDCK) cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Cambrex Bioscience, Walkersville, MD), supplemented with 10% fetal calf serum (FCS, Bodinco Alkmaar, The Netherlands), 100 IU of penicillin/ml and 100 µg/ml of streptomycin (both from Life Technologies, Rochester, NY). The *atg7−/−* and *atg13−/−* knockout U2OS cell lines were knocked out using the CRISPR/Cas9 system and they have been described elsewhere (66). The LC3 TKO and GABA TKO cell lines, and parental sHela cells were kindly provided from Michael Lazarou (67).
Wild type A/PR8 (H1N1) IAV strain was propagated in MDCK cells. The virus titer was determined by measuring the tissue culture infectious does 50 (TCID_{50}) on MDCK cells (68).

4.2 Plasmids and siRNAs

The pCDNA3.1 plasmids carrying the LC3A, LC3B and LC3C proteins were generated by PCR from pDest27-LC3A, pDest27-LC3B and pDest27-LC3C constructs kindly provided from Mathias Faure (69) using appropriate primers and subsequent cloning into the pCDNA3.1 vector using BamHI/XhoI or HindIII/XhoI, creating the pCDN3.1-LC3A, pCDN3.1-LC3B, pCDN3.1-LC3C, pCDN3.1-GABARAP, pCDN3.1-GABARAPL1 and pCDN3.1-GABARAPL2 plasmids. All constructs were verified by DNA sequencing. The pEGFP-LC3B plasmid was kindly provided from Harm Kampinga (70).

The ON-TARGETplus SMARTpool human siRNA targeting LC3A (L-013579-00-0005), LC3B (L-012846-00-0005) and LC3C (L-032399-00-0005) were obtained from GE Healthcare (GE Healthcare, Chicago, IL).

4.3 Immuno-fluorescence analyses

Cells were grown on 12-mm cover slips, transfected and/or infected before being fixed with 4% paraformaldehyde at the indicated times before to be permeabilized in phosphate buffer saline (PBS, 0.137 M NaCl, 0.0027 M KCl, 0.01 M Na$_2$HPO$_4$, 0.0018 M KH$_2$PO$_4$, pH 7.4) containing 0.1% Triton X-100 and 1% of bovine serum albumin (BSA, Sigma, Saint Louis, MO). After blocking with PBS containing 1% FCS, primary antibodies were incubated for 1 h at room temperature and then with a secondary antibody conjugated to either Alexa-488 or Alexa-568 (Molecular Probes, Eugene, OR) (71). The primary antibodies were against LC3 (MBL international, Woburn, MA), GABARAP (a kind gift of Takeda Pharmaceuticals, Osaka, Japan), p62 (Progeny, Heidelberg, Germany), NP (Bio-Rad, Berkeley, CA) and M2 antibody (Abcam, Cambridge, UK). Cells were finally treated with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific) before imaging. Fluorescence signals were captured with a Leica sp8 confocal microscope (Leica, Wetzlar, Germany) or a DeltaVision RT fluorescence microscope equipped with a CoolSNAP HQ camera (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). Quantifications were performed by examining at least 200 cells per experiment.
4.4 Western-blot analyses

Cells grown in 12-well were washed with cold PBS and harvested in 100 µl of 2x Laemmli Sample buffer (65.8 mM Tris-HCl, pH6.8, 26.3% glycerol, 2.1% SDS and 0.01% bromophenol blue) on ice for 30 min, sonicated for 1 min and boiled (71). Equal protein amounts were separated by SDS-PAGE and after western blot, proteins were detected using specific antibodies against IAV NP protein, LC3 (Abgent, San Diego, CA), p62 (Abcam, Cambridge, UK), GABARAP, ATG7 (Cell signaling technology, Danvers, MA), ATG13 (Rockland immunochemicals, Pottstown, PA) and anti-β-actin (Merck Millipore, Massachusetts, US), and secondary antibodies conjugated to Alexa-680 (Molecular probes, Eugene, OR). The signals were captured with an Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE) and the protein signal intensities were quantified using the ImageJ software and normalized to the loading control, i.e. β-actin (72).

4.5 Statistical analyses

Data represent the means of 3 independent biological replicates ± standard deviation (SD). Data were statistically analyzed using the Microsoft Excel software (Redmond, WA) with the paired two-tailed Student’s t test. All comparisons with a p-value <0.05 were considered statistically significant and highlighted with the symbol *. Non-significant differences are not indicated. Images in the figures show representative experiments.

Acknowledgements

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Supplementary figure

A

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![Image](image41)

LC3-positive NP puncta

![Image](image42)
Figure S1. Ectopically expressed GFP-LC3 does not associate with NP puncta in U2OS cells. A. U2OS cells were transfected with GFP-LC3 or not for 48 h and subsequently infected with IAV at a moi of 0.1 before being processed for IF at 16 hpi. The transfected cells were stained with anti-NP or anti-M2 antibodies, while non-transfected cells were stained with anti-LC3 and anti-NP or anti-M2 antibodies. Arrowheads highlight M2 localization to the plasma membrane in the insets. Size bar, 5 μm. B. Quantification of the NP puncta positive for endogenous LC3 or GFP-LC3 in the experiment shown in panel A. Error bars represent the SD of three independent experiments. Asterisks indicate significant differences (p <0.01).
Figure S2. Non-lipidated LC3 associates with IAV NP protein. A. Cell extracts from WT, atg7<sup>−/−</sup> and atg13<sup>−/−</sup> cells were separated by SDS-PAGE and western blot membranes probed with antibodies against ATG7, ATG13, LC3 and β-actin (loading control). B. WT and atg13<sup>−/−</sup> cells were analyzed as in Fig. 4D. Size bar, 5 μm. C. Quantification of the NP puncta positive for LC3 in the experiment shown in panel D. Error bars represent the SD of 3 independent experiments.
Figure S3. GABARAP proteins are not involved in IAV infection. A. Cell extracts from sHela, LC3 TKO and GABA TKO cells were separated by SDS-PAGE and western blot membranes probed with antibodies against GABARAP, LC3 and β-actin (loading control). B. sHela and LC3 TKO cells were processed for IF using anti-LC3 antibodies. Size bar, 5 μm. C. sHela and GABA TKO cells were processed for IF using anti-GABARAP antibodies. Size bar, 5 μm. D. A549 cells were infected with IAV at a moi of 0.1 before being processed for IF at 16 hpi. The cells were stained with anti-NP and anti-GABARAP antibodies. Size bar, 5 μm.