






Manipulation of autophagy by SARS-CoV-2 proteins

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ABSTRACT

As part of innate immune defenses, macroautophagy/autophagy targets viruses and viral components for lysosomal degradation and exposes pathogen-associated molecular patterns to facilitate recognition. However, viruses evolved sophisticated strategies to antagonize autophagy and even exploit it to promote their replication. In our recent study, we systematically analyzed the impact of individual SARS-CoV-2 proteins on autophagy. We showed that E, M, ORF3a, and ORF7a cause an accumulation of autophagosomes, whereas Nsp15 prevents the efficient formation of autophagosomes. Consequently, autophagic degradation of SQSTM1/p62 is decreased in the presence of E, ORF3a, ORF7a, and Nsp15. Notably, M does not alter SQSTM1 protein levels and colocalizes with accumulations of LC3B-positive membranes not resembling vesicles. Infection with SARS-CoV-2 prevents SQSTM1 degradation and increases lipidation of LC3B, indicating overall that the infection causes a reduction of autophagic flux. Our mechanistic analyses showed that the accessory proteins ORF3a and ORF7a both block autophagic degradation but use different strategies. While ORF3a prevents the fusion between autophagosomes and lysosomes, ORF7a reduces the acidity of lysosomes. In summary, we found that Nsp15, E, M, ORF3a, and ORF7a of SARS-CoV-2 manipulate cellular autophagy, and we determined the molecular mechanisms of ORF3a and ORF7a.

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The pandemic severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has emerged at the end of 2019 and rapidly spread worldwide. To date (June 2021), more than 170 million people were infected, and more than 3.5 million deaths were reported. Part of the underlying reason for its unfortunate success is efficient evasion and counteraction of our immune defenses, among them autophagy. Autophagy is an evolutionarily ancient auto-digestive system that has an important role in anti-viral defenses. It is capable of targeting viral components or even full viruses for lysosomal degradation. Thus, most, if not all, successful viruses developed strategies to avoid degradation by autophagy. Some viruses even evolved to exploit components of the autophagic machinery to promote their replication and to mediate membrane trafficking and fusion processes.

In a recent paper [1], we showed that infection of human cells with SARS-CoV-2 reduces autophagic flux. This is characterized by two hallmarks, an accumulation of SQSTM1 and the increased presence of the processed form of LC3B, LC3B-II. Notably, the virus is relatively unaffected by the induction of autophagy with rapamycin but remains sensitive towards innate immune activation via interferons *in vitro*. Thus, SARS-CoV-2 efficiently avoids the anti-viral functions of autophagy. To address the underlying molecular mechanisms, we systematically analyzed the impact of 29 of the 30 known SARS-CoV-2 proteins on autophagy. Our results demonstrate that the number of LC3B-positive autophagosomes is decreased in the presence of Nsp15, whereas E, M, ORF3a, and ORF7a expression causes a strong accumulation of membrane-associated LC3B (Figure 1). To dissect whether this accumulation is due to *de novo* induction of autophagy or

rather caused by a block of autophagic flux, we reexamined the impact of these proteins on autophagosome numbers either in conditions of induction or blockage of autophagic flux. Whereas induction of autophagy by rapamycin does not dramatically alter the effects of E, M, ORF3a, and ORF7a, bafilomycin A₁ masks their effects, indicating that these viral proteins prevent autophagic flux. Notably, the decrease of autophagosomes observed for Nsp15 is alleviated by rapamycin treatment, suggesting that Nsp15 affects the MTOR axis. Western blot analysis confirmed these results. In the presence of E, M, ORF3a, and ORF7a, processed LC3B-II accumulates, whereas expression of Nsp15 causes a slight but consistent decrease in LC3B-II. In line with this, E, ORF3a, ORF7a, and Nsp15 expression causes accumulation of SQSTM1. Notably, although M induces an increase of LC3B-membrane localization and LC3B processing, it does not inhibit SQSTM1 degradation, indicating that this viral protein does not block classical autophagy. Subsequent immunofluorescence analysis revealed that while E, ORF3a, and ORF7a induce an increase of LC3B-positive vesicle-like puncta upon overexpression, M localizes to large LC3B-positive accumulations. Nsp15 reduces the number of autophagosomes per cell. Notably, our analysis further showed that the function of SARS-CoV-2 ORF3a, ORF7a, M, and Nsp15 in autophagy seems to be conserved in the closest related bat coronavirus RaTG13 and the previous epidemic SARS-CoV-1.

To understand the molecular mechanisms by which ORF3a and ORF7a block autophagic flux, we performed proteome analysis. The gene ontology analysis of regulated proteins revealed that a major compartment targeted by the two accessory proteins are late endosomes. In line with this, both ORF3a and ORF7a,

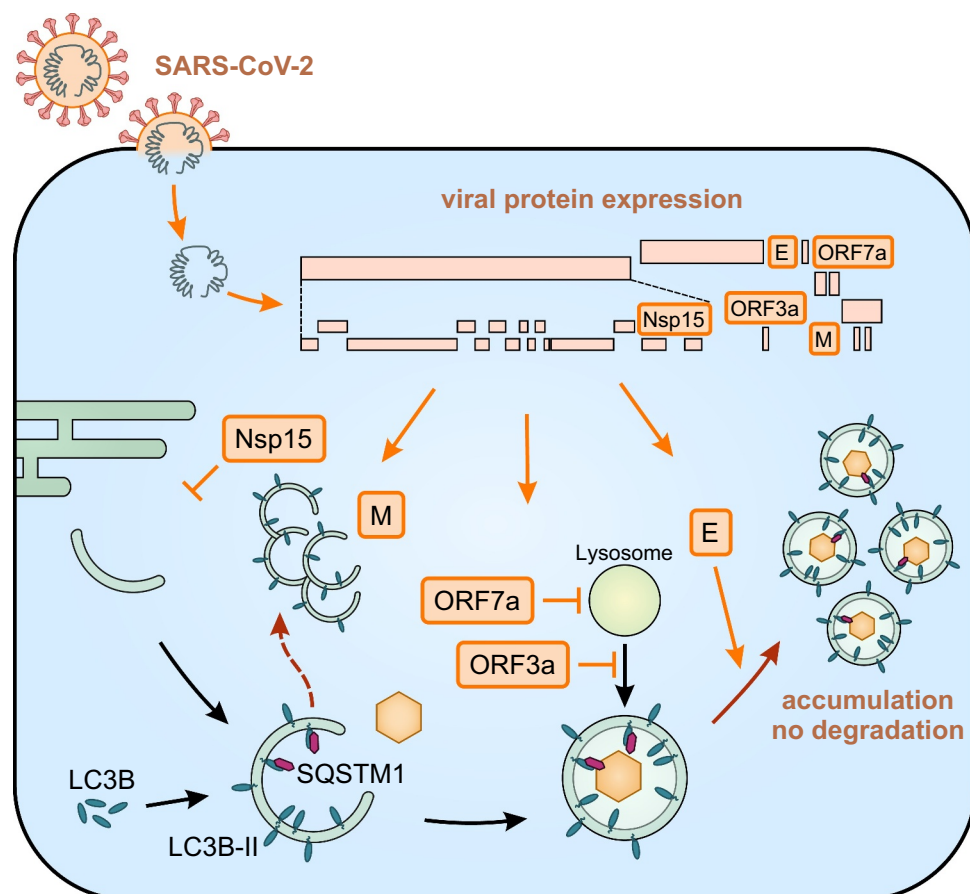


Figure 1. The impact of SARS-CoV-2 proteins on autophagy.

After nucleation, the autophagosome is elongated and decorated with LC3B-II. SQSTM1 is an autophagy receptor that recruits cargo. Infection of cells by SARS-CoV-2 leads to the expression of viral proteins (orange boxes) and autophagy is substantially regulated by Nsp15, M, ORF7a, ORF3a, and E. Altogether, infection with SARS-CoV-2 leads to an accumulation of autophagosomes and prevents degradation of their cargo.

colocalize with late endosomal markers and trans-Golgi network markers, but less so with early endosomal markers. Autophagosome maturation can be analyzed using pH-sensitive molecular probes. Using an mCherry-GFP-LC3B reporter system, we showed that both ORF3a and ORF7a prevent the acidification of autophagosomes. Further experiments revealed that ORF7a reduces the number of acidic lysosomes, whereas ORF3a leads to their accumulation. This indicates that ORF7a reduces lysosome acidity and thereby autophagosomal degradation, whereas ORF3a prevents fusion between lysosomes and autophagosomes. In line with this, lysosomal markers and autophagosomal markers colocalize in the presence of ORF7a, whereas this co-localization is reduced by ORF3a. While our results revealed the molecular mechanisms of ORF3a and ORF7a in autophagy, further studies are required to understand how E, M, and Nsp15 modulate autophagy.

As SARS-CoV-2 seems to decrease autophagic flux, the inhibition of autophagy by Nsp15 seems counterintuitive at first. However, several viruses, such as HIV-1, inhibit both induction and turnover of autophagosomes, indicating that a two-pronged strategy to avoid autophagy may be required. Our results suggest that M may not regulate classical autophagy, but rather recruit LC3B-II-positive membranes to perinuclear assemblies. It is tempting to speculate that these could constitute double-membrane structures required for proper replication of SARS-

CoV-2. Budding of SARS-CoV-2 also requires components of the autophagic/lysosomal pathway. Thus, ORF7a-mediated deacidification of lysosomes may additionally promote virion release by avoiding degradation. Further, the structural protein E may also play a role in redirecting autophagosomes to facilitate SARS-CoV-2 virion release via the lysosomal route. Notably, both E and M are part of the incoming virion, thus upon entry they may already manipulate autophagy to avoid rapid targeting and degradation.

Some of the viral proteins that modulate autophagy have enzymatic functions. For example, Nsp15 is an endoribonuclease. These functions may be required for their interference with autophagy, and targeting them could prove to be an opportunity for novel anti-SARS-CoV-2 strategies.

We showed that the proteins of the closest related bat CoV, RaTG13, interfere with human autophagy equally well as their SARS-CoV-2 counterparts. It will be interesting to determine whether more distantly related bat CoVs are also able to counteract human autophagy. Rapid full sequence analysis during this pandemic additionally provides the opportunity to monitor adaptations to autophagy while this virus spreads in the human population. Notably, ORF3a seems to be a hot spot for mutations.

In summary, our study provides an overview of the proteins used by SARS-CoV-2 to manipulate autophagy (Figure 1) and gives mechanistic insight on how ORF3a and ORF7a interfere

with autophagic flux. This may help to understand the pathogenesis of SARS-CoV-2 and its resistance towards autophagy induction. In addition, our results also provide a basis for future studies on the molecular mechanisms of E, M, Nsp15 and dissecting the contribution of autophagy modulation by individual proteins to SARS-CoV-2 replication.

Disclosure statement

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