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Pandemics of severe acute respiratory infections have been serious threats to global health, causing significant morbidity and mortality (1). In particular, influenza A virus (IAV) and coronaviruses (CoV) such as Severe Acute Respiratory Syndrome (SARS-CoV) and Middle East Respiratory Syndrome CoV (MERS-CoV), have caused minor to major outbreaks of viral pneumonia worldwide despite advances in their treatments. Consequently, it is crucial to study for in-depth of these viruses life cycle to better understand how they interact with cellular pathways and thus potentially develop more effective therapies.

Although the symptoms of the diseases caused by these viruses are similar and include fever, cough, and shortness of breath, IAV and CoV belong to two distinct virus families (1). The research described in this thesis focused on these two viral families with first visualization of the ultrastructure of the membrane rearrangements induced by an established model virus for α-CoV, porcine epidemic diarrhea virus (PEDV), in host cells (Chapter III). We then focused on the N protein of mouse hepatitis virus (MHV), a prototype virus for β-CoV, in infected cells to characterize the function and the relevance of this protein in the viral life cycle (Chapters IV and V). Next, we investigated the interplay between IAV and autophagy. Eventually, we demonstrate that one of the ATG8 protein subfamilies, the one comprising the LC3 proteins, has an autophagy-independent role in IAV replication (Chapter VI).

1. Summary

1.1 Intracellular membrane rearrangements induced by α-CoV

Infection of mammalian cells with CoV triggers the vesiculation, the reorganization and the rearrangements of intracellular membrane structures. These modifications of the host cell compartments have been shown to be important in promoting the replication and egression of CoV (2-6). They have been principally investigated in the context of β-CoV and γ-CoV infections, but they remain largely unexplored in other CoV sub-families and might differ (4-13). In chapter III, we thus focused on PEDV, an α-CoV. In particular, we characterized the intracellular membrane reorganization at the ultrastructural level over the course of the PEDV infection by qualitative and quantitative electron microscopy examinations.

We found that similar to most of the CoVs belonging to other virus sub-families (5, 6, 8, 13), PEDV initially induces ER proliferation to form numerous double-membrane vesicles (DMVs) and the network of reticular inclusion,
which was previously named convoluted membranes (CMs). These membranous rearrangements are probably necessary to create a scaffold for the assembly of viral replication-transcription complexes (RTCs) (Chapter III, Fig. 1). Interestingly, the localization, number and morphology of DMVs changed over the course of PEDV infection, from a few regular and circular DMVs at the early stages, to numerous large clusters distributed to the perinuclear region at late stages (Chapter III, Fig. S2). It has been proposed that CMs are the generation sites for DMVs (6). As a matter of fact, CMs and DMVs are composed by the same viral proteins and it has been indicated that CMs are also involved in the replication and transcription of viral RNA in SARS-CoV-infected cells (6). Surprisingly, we observed that CMs appear at the same time as DMVs, but with lower frequency, and CMs were always observed in between or around the cluster of DMVs (Chapter III, Fig. 1B). Therefore, another possible scenario evoked by our study on PEDV is that CMs could originate from the DMVs as appearing at the same time or later, as previously suggested for MHV (5).

We also observed altered Golgi compartments, which appear as large clusters of vacuoles filled with numerous luminal virions. These large virion-containing vacuoles (LVCVs) originate by expansion of the Golgi to accommodate an increased viral particle formation (Chapter III, Fig. 4). In general, CoV assemble in the ERGIC/Golgi, as inferred from the current literature on most of the β-CoV, including MHV and SARS-CoV (5, 14). In PEDV-infected cells, however, we also detected viral particles forming on the limiting membrane of the ER (Chapter III, Fig. 1C-1F). These data are in agreement with what was observed with another α-CoV, transmissible gastroenteritis CoV (TGEV), suggesting a unique characteristic of this CoV genus: viral particles assemble in both the ER and Golgi.

1.2 The association of MHV N protein to RTCs

Following infection and genome translation, positive-stranded RNA viruses establish cytoplasmic enzymatic complexes, called RTCs, which direct the amplification and expression of the viral genome. The viral RNA-dependent RNA polymerase is the central enzyme of RTCs, which also includes viral non-structural proteins (nsp) and host factors that cooperate to synthesize viral RNA (15). A number of studies have implied that the CoV N protein associates with RTCs (5, 16-21). The N nucleocapsid is the only structural protein synthesized in the cytosol and carries out multiple functions in the CoV life cycle (22). In Chapters IV and Chapter V, we characterized MHV N protein in greater molecular detail. The self-interaction of CoV N proteins plays an essential role
in viral particle assembly (23-27). Although the crystal structure of full length N protein has not been resolved yet, our data revealed that the MHV N protein forms large cytoplasmic homo-oligomers during viral infection (Chapter IV, Fig. 2). CoV N proteins entwine the viral genomic RNA (gRNA) and they are thus essential for the incorporation of the viral genetic material into virions (20, 28, 29). Surprisingly, the oligomerization of N protein does not depend on its binding to gRNA (Chapter IV, Fig. 2B-2C), which underlines that this self-interaction might not occur simultaneously with the RNA entwining step, or it utilizes different domains of the N protein. In support of this finding, we showed that two different domains mediate N protein oligomerization and the amino acids crucial for the N protein self-interaction are discontinuously distributed (Chapter IV, Fig. 3). Consistent with our findings, a recent cryo-EM analysis of MHV ribonucleoprotein complexes showed that the N protein forms octamers (30), which subsequently assemble into larger oligomeric structures that could acquire either a loose or a more compact filament shape. Therefore, we hypothesized that the N protein first oligomerizes and then is recruited to the RTCs at DMVs and/or CMs, to allow the efficient and tight loading of the exceptionally large gRNA into a ribonucleoprotein complex (Chapter IV, Fig. 5).

CoV replicase gene products are processed into single non-structural protein (NSP) by one 3C-like protease (3CLpro) and two viral papain-like proteases (PLpro). The PLpro activity resides with nsp3, which is a 213 kDa multidomain polypeptide that is integrated into membranes (31). In addition to its involvement in proteolytic processing, the PLpro also acts as a de-ubiquitination enzyme subverting the cellular ubiquitination machinery to facilitate viral replication (32). The transmembrane domains within nsp3, nsp4 and nsp6 are predicted to be required as integral membrane scaffolding components that facilitate assembly and localization of the RTCs (33). A number of studies have shown that CoV N protein dynamically associates with RTCs, implying that the N protein could interact with one or more NSPs (5, 16-19). At the RTCs, the N protein could stimulate the synthesis of viral RNA, mRNA and gRNA, and eventually promote the gRNA package with N oligomers to form ribonucleoprotein complexes. Our interactome study has revealed that MHV N protein principally binds to nsp3 (Chapter V, Fig. 1B), an interaction also found by others (21, 34-36).

The role of N protein over the course of infection, as proposed in Chapter IV based on our results, is that it constitutively assembles into oligomers and those are recruited to the RTCs. This latter step is mediated by the interaction
with nsp3 but it does not require the binding to gRNA (Chapter V, Fig. 4). In addition, blocking of N-nsp3 interaction leads to a defect in both the MHV infection and viral RNA synthesis in vitro (Chapter VI, Fig. 6 and 7). Taken together, our data reveal that the recruitment of N protein to RTCs plays an essential role in MHV life cycle by promoting the replication of this virus.

1.3 LC3 proteins are required for IAV infection

Autophagy is a conserved intracellular catabolic pathway that allows cells to maintain homeostasis through the degradation of deleterious components via autophagosomes (37). Induction of autophagy upon IAV infection is considered a necessary event for the normal progression of the life cycle of this virus (38-43), which however also inhibits autophagosome fusion with lysosomes (40). In Chapter VI, our initial aim was to better understand the interaction between IAV and the autophagy machinery.

Growing evidence suggests that IAV infection increases the generation of autophagosomes (38-40, 44). Our studies confirm the finding that IAV indeed triggers autophagy in host cells (Chapter VI, Fig. 1-3). To better understand whether this degradative pathway contributes to IAV replication, we infected autophagy deficient cells, i.e. atg7−/− and atg13−/− knockout cells. Interestingly, we found that an intact autophagy machinery is dispensable for IAV infection as ATG7 or ATG13 are not necessary for viral infection (Chapter VI, Fig. 4).

It has been shown that several pathogens have evolved strategies to evade the autophagy pathway or even manipulate it to sustain their intracellular life cycle (45-47). Autophagy is orchestrated, step by step, by multiple autophagy-related (ATG) proteins (48). In addition to their traditional role in autophagosome formation, recent findings have shown that ATG proteins function in other pathways as well (49). For example, ATG proteins have been shown to assist macroendocytic engulfment and degradation of dead cells as well as pathogens (50). In Chapter VI, while investigating the interplay between IAV and ATG proteins, we found that LC3 associates with viral ribonucleoproteins (vRNPs) during IAV infection and that the lipidated form of LC3 is not required for this association (Chapter VI, Fig. 1-3). The mammalian ATG8 protein family is subdivided into LC3 and GABARAP protein subfamilies (51, 52), and we demonstrate that the LC3 proteins, but not the GABARAP proteins, associate with vRNPs and play a crucial role in IAV infection (Chapter VI, Fig. 5). These findings therefore strengthen our previous conclusion that an intact ATG
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machinery is not required for IAV infection but instead suggest that specific ATG proteins might assist IAV infection in an unconventional way.

2. Concluding remarks and perspective

Data presented in this thesis aid to decipher import aspects of the life cycle of viruses belonging to the virus families that cause severe acute respiratory infections. In particular, we have characterized the intracellular membrane rearrangements and the relevance of the N protein interactions at the early steps of the CoV life cycle, and unveiled an autophagy-independent role of LC3 proteins in IAV infection.

In this context, we have shown that the blocking of N-nsp3 interaction impairs the viral life cycle by probably affecting optimal viral RNA synthesis and thus also subsequent transcription of viral factors. However, the mechanism between N-nsp3 interaction at RTCs and subsequent RNA transcription on the ER remains unclear. A possible mechanism is that the N protein promotes the viral RNA transcription by interacting with transcriptional regulatory sequences (TRSs) (53), which are present on the gRNA and are required for expression of each viral gene. Another option is the N-nsp3 binding at RTCs enhances the synthesis of viral RNA by possibly allosterically stimulating enzymatic activities and/or providing a more structured conformation of the complex that positively influences protein and RNA interactions. In this regard, additional future investigations are needed to dissect the protein-protein and protein-RNA interactions that are essential for CoV RNA synthesis.

CoV N proteins are the only structural proteins that associate with the viral gRNA and it is still unclear whether the gRNA entwining around the N protein oligomers takes places at RTCs or in a subsequent step. One scenario could be that the gRNA associates with the N oligomers co-transcriptionally or shortly after gRNA synthesis at the RTCs, and nsp3 could play a key role in coordinating these events. The second scenario is that the CoV ribonucleoprotein complexes are formed in the cytoplasm or while virions are being assembled at the ERGIC/Golgi. The hierarchical order of the interactions between the N protein, gRNA and the rest of the structural proteins, needs further investigation, which is critical to understand in detail the CoV infection at the molecular level.

Although our studies focused on MHV, this virus is an established model for the investigation of β-CoV, which include human pathogens such as SARS-CoV and MERS-CoV. Currently, there is no effective cure for SARS-CoV and MERS-CoV, and insights into steps of the viral life cycle that could be targeted
by developing specific drugs are needed to fight possible novel emergent CoV epidemics (54). The N protein is the most abundant CoV protein and it is essential for the life cycle of these viruses. Mutations of the binding domains mediating the MHV N protein-nsp3 interaction that we described in this thesis, block vRNA synthesis. Future in vitro and in vivo studies must determine whether this also applies to SARS-CoV and MERS-CoV. As this knowledge will be key to the design of antiviral drugs specifically targeting N-nsp3 binding.

By characterizing the relationship between IAV and LC3 proteins, we have discovered that not all the ATG8 proteins are essential for IAV infection, i.e. only LC3 proteins are crucial for the IAV infection. The RNA polymerase, as well as multiple copies of viral NP protein, are the main components of vRNPs. vRNPs are newly assembled in the nucleus and subsequently transported into the cytoplasm, where they accumulate near the microtubule-organizing center. The question of whether LC3 proteins participate in the transport of vRNPs in the host cell and whether they function by interacting with NP, viral RNA polymerase or vRNA, remains to be investigated. Other functions, however, cannot be excluded. Moreover, LC3 proteins could bind vRNPs indirectly via host factors. For example, LC3 binds to the tumor protein 53 inducible nuclear protein 2 (TP53INP) (55) and together with high mobility group box 1 (HMGP1), translocates from the nucleus to the cytoplasm upon starvation and participates in regulation of autophagosome biogenesis and protein degradation (56, 57). Interestingly, HMGP1 also interacts with IAV NP to promote the viral replication in the nucleus (58) and therefore, LC3 might already bind to vRNPs in the nucleus via TP53INP2/HMGP1. On the other hand, a LC3-interacting region (LIR) motif (59) might be presented in one or more components of vRNP and mediated their interaction with LC3 proteins.

This study underlies that LC3 proteins are essential for IAV infection and further investigations unveiling the mechanism of LC3 binding to vRNPs could pave the way to study the autophagy-independent role of LC3s in IAV infection. New influenza IAV strains continue to arise every year. An estimated 900,000 people had symptomatic influenza and more than 9,500 died from October 2017 to May 2018 in Netherlands (60). In this context, further studies must explore whether the mechanism of LC3-vRNP binding is conserved between different strains in vitro, in cell cultures. This information will provide possible insights into the design of candidate broad antiviral therapeutics for IAV, which specifically target LC3-vRNP binding. The identification of the proteins and the domains involved in LC3-vRNP interaction will need to be studied in vitro.
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before creating inhibitors and test them \textit{in vivo}, possibly using some of the methods described in this thesis, and in animal models.

The transmission of respiratory viral infections is much more rapid and easier than that of most of other viruses. The work presented in this thesis aimed at dissecting specific steps of the viral life cycle and their implications in the biology of the studied viruses. Our findings provide solid bases for future investigations on the molecular mechanisms underlying determined interactions and may allow us to developing novel of therapeutic approach to fight respiratory viral infections.
Reference:


