Molecular insights into viral respiratory infections
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CHAPTER I

General Introduction
Chapter I

The structure of the respiratory tract facilitates gas exchange between the exterior environment and interior milieu of the host, while it is a susceptible target and feasible gateway for diverse pathogens. Pandemics of severe acute respiratory infections have been serious threats to global health, causing significant morbidity and mortality. In particular, influenza viruses and coronaviruses (CoV), including MERS-CoV and SARS-CoV, have caused numerous outbreaks of viral pneumonia worldwide with different impacts. To survive and propagate in the cells, viruses and pathogens usurp multiple host pathways to replicate and egress from the host cells. There are, however, still numerous critical questions regarding the cell biology of these viruses and their understanding could provide the framework for the development of novel therapeutic strategies. Autophagy is a conserved intracellular pathway that allows cells to maintain homeostasis through degradation of deleterious components via specialized double-membrane vesicles called autophagosomes. During the past decades, it has been revealed that numerous microbes, including viruses, hijack autophagy in order to promote their life cycle. In this thesis, we have focused on acquiring new molecular insights into the viral replication of CoV, and investigated the relationship between influenza A virus (IAV) and autophagy.

1 The Coronavirus

1.1 General characteristics of Coronaviruses

CoV are a member of the *Coronaviridae* virus family. They are enveloped viruses, with positive single stranded RNA genomes. For a detailed introduction about their pathogenicity and life cycle, refer to Chapter II.

1.2 Life cycle of Coronaviruses

1.2.1. Entry, replication and transcription

The entire CoV replication cycle takes place in the host cell cytoplasm. The initial attachment of the virion to the host cell is initiated by interactions between the spike protein (S) and its receptor. The receptor binding domains (RBD) are localized within the S1 region of a CoV S protein, with some viruses having the RBD at the N-terminus of S1 (e.g. MHV) while others (e.g. SARS-CoV) having the RBD at the C-terminus of S1 (1, 2). The S protein-receptor interaction is the primary determinant for CoV to infect a host species and also governs the tissue tropism of the virus (3). Many CoV utilize peptidases as their cellular receptor
It is unclear why peptidases are used as receptors, as entry occurs even in the absence of the enzymatic domain of these proteins.

Following receptor binding, the virus has to gain access to the host cell cytoplasm (Fig. 1, step 1). This is generally accomplished by acid-dependent proteolytic cleavage of S protein by proteases, followed by fusion between viral and cellular membranes (5). Fusion generally occurs within acidified endosomes, but some CoV, like mouse hepatitis virus (MHV) or transmissible gastroenteritis coronavirus (TGEV), can fuse at the plasma membrane (6). Cleavage of the S protein exposes a fusion peptide that inserts into the endosomal membrane, which is followed by the joining of two amino acid heptad repeats to form an antiparallel six-helix bundle that allows mixing of viral and cellular membranes, resulting in fusion and ultimately release of the viral genome into the cytoplasm (6).

The next step in the CoV life cycle is the translation of the replicase gene from the viral genomic RNA. The replicase gene encodes for two large transcripts, ORF1a and ORF1b, which express two co-terminal polyproteins, pp1a and pp1ab (Fig. 1, step 2). These polyproteins self-process into 11 and 16 individual non-structural proteins (NSPs), respectively (7). CoV encode either two or three proteases that are involved in the cleavage of these two replicase polyproteins. They are the papain-like proteases (PLpro), present within nsp3, and a serine protease or Mpro, localized in nsp5 (8). Most CoV encode two PLpros within nsp3, except gamma-CoV, SARS-CoV and MERS-CoV, which only express one PLpro (8). The NSPs generated by the self-processing of pp1a and pp1ab, subsequently assemble into replicase transcriptase complexes (RTCs) to create an environment suitable for RNA synthesis, which is responsible for RNA replication and transcription of sub-genomic RNAs (9-11) (Fig. 1, step 3). CoV replicative platforms consist of cytoplasmic double-membrane vesicles (DMVs) into which the RTCs are anchored (12-14). Studies have shown that DMVs are integrated into a reticulovesicular network of modified endoplasmic reticulum (ER) membranes, also referred to as convoluted membranes (CMs) (15).

Viral RNA synthesis follows the translation and assembly of viral RTCs and produces both genomic and sub-genomic RNAs (16, 17) (Fig. 1, step 4). Sub-genomic RNAs serve as mRNAs for the structural and accessory genes, which reside downstream of replicase polyproteins. All positive-sense sub-genomic RNAs are 3’ co-terminal with the full-length viral genome and thus form a set of nested RNAs. Both genomic and sub-genomic RNAs are produced through
Figure 1. Schematic overview of the CoV life cycle. Infection begins with the binding of the viral particles to cell surface receptor and subsequent entering in the cell through fusion with the endosome membrane upon endocytosis (step 1). Translation of the released genomic RNA (gRNA) yields replicase polyproteins, i.e., polyprotein 1a (pp1a) and polyprotein 1ab (pp1ab) (step 2), which undergo proteolytic self-processing to generate the NSPs that assemble into replication-transcription complexes (RTCs). The RTCs are part of membranous network composed of double membrane vesicles (DMVs) and convoluted membranes, which engage in minus-strand RNA synthesis to produce minus-strand RNAs (step 3). Subsequently, they produce the gRNA and plus-strand sg mRNAs (step 4), which is required for the expression of the structural protein genes. Newly synthesized S, E, and M proteins are inserted in the endoplasmic reticulum (ER) whereas the N nucleocapside is cytoplasmic and interacts with RTCs (step 5) to associate with the gRNA to form ribonucleoprotein complexes. Virion assembly takes place in the ER-Golgi intermediate compartment (ERGIC) (step 6), and involves the inward budding of the limiting membrane of these compartments, which is triggered by the interaction between the transmembrane structural proteins and the ribonucleoprotein complex. Finally, mature virions egress through the exocytosis (step 7).
negative-strand intermediates (17).

1.2.2. Virion assembly and release

Following replication and sub-genomic RNA synthesis, the viral structural proteins, S, E, and M are translated and inserted into the ER (18, 19) (Fig. 1, step 5). These proteins move along the secretory pathway to the ER-Golgi intermediate compartment (ERGIC) (18, 19). There, they interact with N proteins associated with genomic RNA, to trigger the inwards budding of ERGIC limiting membrane that leads to the formation of intralumenal mature virions (20). The M protein directs most protein-protein interactions required for the assembly of these CoV viral particles (21). The M protein, however, is not sufficient for virion formation, as virus-like particles (VLPs) cannot be formed by M protein expression alone (22). In contrast, when M protein is co-expressed with E protein, VLPs are formed suggesting that these two proteins function together to produce CoV envelopes (22). N protein enhances VLPs formation, indicating that encapsidated genomes enhance viral envelopment at the ERGIC (23). The S protein is incorporated into virions at this step, but is not required for assembly (24). The ability of the S protein to traffic to the ERGIC and interact with M protein is critical for its incorporation into virions (24) (Fig. 1, step 6).

Following assembly, virions are transported to the extracellular milieu in exocytic vesicles and released by secretion (16, 20) (Fig. 1, step 7). It remains unknown whether virions use the traditional pathway for transporting of large cargo from Golgi or whether virus has devised a separate, unique pathway for its own exit. Over the course of CoV infections, the excess S protein that does not get incorporated into virions, traffics to cell surface where it mediates cell-cell fusion between infected cells and adjacent, uninfected cells (16, 25, 26). This leads to the formation of giant, multinucleated cells, which allows virus to spread within an infected tissue/organism without being detected or neutralized by immune system.

2 Influenza A virus

2.1 General characteristics of Influenza A virus

Influenza is an acute respiratory illness that has been recognized since 16\textsuperscript{th} century and spreads rapidly through communities in outbreaks (27, 28). Influenza viruses are enveloped RNA viruses belonging to the relatively small Orthomyxoviridae virus family (27, 28). The single-stranded genomic RNA of this family has been termed as negative in contrast to mRNA which, by
convention, is considered positive-strand RNA (29). At present, the Orthomyxoviridae virus family consists of five genera: Influenza A, Influenza B, Influenza C, Thogovirus, Influenza D, Quaranjavirus and Isavirus (30). The first four genera were established long time ago (31). Isavirus is a relatively new addition to Orthomyxoviridae, it contains an infectious salmon anemia virus, which is a devastating pathogen in fish farms all over the world (31). The Influenzavirus D and Quaranjavirus, predominantly infect animals, in particular cattle and arthropods or birds, respectively (31-34). From the epidemiological point of view, the Influenzavirus A genus is the most important for humans. The only member of this genus, influenza A virus (IAV) is sub-categorized into multiple subtypes. According to current nomenclature, description of subtypes includes the host from which it was originally isolated, geographic location of first isolation, strain number, year of isolation and in parentheses, the serological characteristics of two viral proteins: hemagglutinin (HA) and neuraminidase (NA). For the strains isolated from humans, the name of the host is omitted (35, 36). For example, A/California/7/2009 (H1N1) indicates human influenza type A, isolated initially in California, strain number 7, year of 2009, type H1N1. The A/Turkey/England/91(H5N1) name is for influenza type A, isolated from turkeys in England in 1991, type H5N1.

IAV virus genome is composed of eight segments of single-stranded RNA (ssRNA) of negative sense and consists of approximately 14,000 nucleotides, which are named segment 1 to 8, and encodes for PB2, PB1 and PB1-F2, PA and PA-X, HA, NP, NA, M1 and M2, and NS1 and NS2/NEP, respectively (37). Only segment 8 encodes non-structural proteins, i.e. NS1 and NS2/NEP (38). The organization of eight RNA segments inside the virions is not fully understood. Electron microscopy studies indicate that the NP protein, which forms helical structures, enwraps each RNA segment individually (39-41).

Orthomyxovirus virions are usually almost spherical, with a diameter ranging from 100 to 200 nm (29). However, some laboratory strains form extended threadlike structures, which sometimes exceed 1000 nm in length (42, 43). Virions are relatively unstable in the environment and influenza viruses are easily inactivated by heat, dryness, pH extremes and detergents (44). The outer lipid bilayer of influenza viruses originates from plasma membranes of the host in which the virus propagates (45). Around lipid envelope of viral particle, there are approximately 500 projections in the form of spikes (46). About 80% of these projections are composed by HA and resemble rods (46, 47). The remaining projections are in the shape of mushrooms and they are built by NA molecules (47). The viral outer membrane also contains some copies of the small M2
proteins that form ion channels in virion particles (48, 49). Matrix protein M1, which is the most abundant protein in virions, underlines the lipid layer and plays an important role in the attachment of the ribonucleoprotein (RNP) during virion formation (50, 51). RNP core is a complex structure composed mostly by NP protein, which enwraps the eight different RNA segments of the IAV genome (52, 53) (Fig. 2A). Additionally, RNPs contain about 50 copies per virion of RNA-dependent RNA polymerase, which in case of IAV is a complex composed of three proteins: PB1, PB2 and PA (54, 55) (Fig. 2A).

2.2 Life cycle of Influenza A virus

2.2.1 Entry, replication and transcription

Influenza A virus utilizes sialic acid residues on the surface of cells as viral receptors to enter host cells (27, 44, 56) (Fig. 2B, step 1). The HA of influenza viruses replicating in different species shows some preference for particular glycosidic linkages of sialic acid. Human viruses’ HA molecules bind very well to sialic acids conjugated to galactose by alpha-2,6-glycosidic bonds (57). On the other hand, avian viruses preferentially associate to sialic acids connected with galactose by alpha-2,3-glycosidic bonds (57). Alpha-2,6 linkages of sialic acids to galactose are mostly found in human trachea, while sialic acid-alpha-2,3-galactose sugar moieties are principally present in the gut epithelium of birds (57). These receptor distributions explain the preference for selected hosts but also not absolute specificity, which may be bypassed by high viral inoculums or point mutations in the HA gene (29).

After binding to the receptor, virions are internalized through trafficking via the endocytic pathway (58) (Fig. 2B, step 2). The main mechanism is clathrin-mediated endocytosis, although a non-clathrin, non-caveolae pathway has also been attributed to influenza entry (59, 60). HA is synthesized as a precursor HA0 and needs to be cleaved by a host cell protease into the subunits HA1 and HA2 to gain its fusion capacity (61-63). Proteolytic cleavage of HA0 enables HA to undergo conformational changes at low pH, which expose the N-terminal hydrophobic fusion peptide of HA2 and trigger membrane fusion (64). In particular, this protein fragment gets anchored into the internal surface of endosomal membrane while the rest of HA remains tightly associated to the viral envelop via its transmembrane segment (65, 66). A drop of pH in endosome acts as the main trigger for HA2 as a large conformational change that leads to anchoring of the fusion peptide, which is followed by close contact between the adjacent membranes and the subsequent fusion (67, 68).
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As a consequence of these processes, virion content, in particular RNP complexes, is released into cytoplasm of the host cell (69, 70) (Fig. 2B, step 3). Apart from the HA, M2 protein also plays an important role in the release of genetic material into the host cell. This ion channel mediates the influx of H+ ions from the endosomal lumen into the viral particles, which greatly weaken the interaction between RNP complexes and matrix M1 protein (71-73).

Chou et al suggest that all eight segments are transported as one moiety to the nucleus and synthesis of influenza virus RNA occurs there (74). Genomic segments of the virus never exist as independent RNA molecules but rather remain associated with the NP protein (29, 75, 76). RNP complexes, however, are not able to diffuse passively through nuclear pores and consequently they rely on cellular mechanisms of nuclear transport (77). Proteins belonging to the family of alpha-importins recognize nuclear localization signals present in the NP protein and thus play an important role in intranuclear transport of RNP complexes (77).

Once in the nucleus, genomic ssRNA is used as a template for the synthesis of two classes of positive-stranded ssRNA: viral messenger RNAs (vmRNA) and full-length complementary copies (cRNA) (Fig. 2B, step 4 and 5). During transcription, multimeric viral polymerase interacts with the host polymerase II (78). This interaction leads to the phenomenon called “cap-snatching”, which entails removal of a cap from newly synthesized cellular mRNAs (79-81). The PB2 subunit of the viral polymerase is responsible for cap binding (82). The PB1 subunit was regarded as an endonuclease cleaving the 5’ capped oligonucleotide for a long time, which serves as a primer for initiation of transcription (83). Recent crystallographic data, however, suggest that endonuclease activity should be rather attributed to the PA subunit (84). This is one of the observations that contradict the theory that particular activities of polymerase can be dissected and attributed to a single polymerase subunit. It is rather more likely that three subunits of viral polymerase act cooperatively in synthesis of RNA. Transcription continues until approximately 15 nucleotides from the 5’ end of the vmRNA (85). The next step is replication of influenza genomic segments from the template positive-strand cRNAs. This process also generates full-length genome segments that assemble with NP and polymerase subunits to form the RNP complexes, which are subsequently exported into the cytoplasm (86, 87).
Figure 2. Overview of the IAV life cycle. (A) The structure of viral ribonucleoprotein (vRNP) complex. The influenza polymerase is a complex composed by the PA, PB1, and PB2 proteins. One polymerase heterotrimer is attached to each vRNP segment inside the virion and viral RNA molecules (vRNA) are coated by NP molecules. (B) The IAV life cycle. Virus infection is initiated by virus binding to sialylated host cell-surface receptors, which is subsequently followed by endocytosis (step 1). In the host cell, fusion of viral and endosomal membranes occurs at low pH (step 2) and this allows the release of the segmented viral genome into the cytoplasm (step 3). The viral genome is then translocated into the nucleus, where it is transcribed (step 4) and replicated (step 5). Following synthesis in the cytoplasm, viral proteins are assembled into viral ribonucleoproteins (vRNPs) in the nucleus. Export of vRNPs to the cytoplasm is mediated by M1 and NS2. Viral particles are then assembled at the plasma membrane (Step 6), and access the extracellular milieu through budding (step 7).
Chapter I

2.2.2 Virion assembly and release

It has been postulated that assembly of IAV viral components occurs preferentially in lipid rafts present at plasma membrane, a notion emerged from the examination of the lipid content of purified viral particles (88-90). Additionally, HA and NA glycoproteins are incorporated into lipid rafts (91-93). Before reaching the lipid rafts, however, these two glycoproteins are post-translationally modified. These modifications take place in ER where these proteins become correctly folded and glycosylated. They are also assembled into oligomers, i.e. HA into trimers whereas NA into tetramers. Subsequent modifications of the glycan chains of these proteins and their esterification with fatty acids occur in Golgi apparatus (94). Finally, the HA and NA oligomers reach plasma membrane. Signals for the association of these glycoproteins with lipid rafts are located within their transmembrane domains (95). RNPs are probably transported to the region of assembly at apical plasma membrane independently of other structural proteins (Fig. 2B, step 6). The packaging of the eight different RNA segments in the form of RNPs into virion shells is a phenomenon poorly understood, although many models have been proposed (96-98). Some evidences suggest that presence of packaging signals at both ends of the genomic segments, but the precise sequences and/or structures responsible for this encapsulization are still not well defined. Both NA and HA have affinity for sialic acid residues but they have opposite effects on the release of the virus from cells. HA anchors the virus to the cell membrane via its interaction with sialic acid-containing receptors. NA, in contrast, removes sialic acids in order to allow the virion to leave its host cells. Therefore, there must be a coordinated balance between the activities of these two glycoproteins, to maximize production of the virus progeny (29). Finally, virus release requires completion of budding event through fusion of the opposing membranes, which leads to the closure of the viral particle and its concomitant separation from the host plasma membrane (Fig. 2B, step 7).

3 Autophagy

3.1 The mechanism of autophagy and the autophagy-related proteins

Cellular homeostasis requires a proper balance between anabolism and catabolism. The two major cellular degradative pathways in eukaryotic organisms are proteasome and autophagy. Autophagy is a cellular process of self-digestion, in which cells capture their own cytoplasm and organelles, and consume them in lysosomes (99, 100). There are distinct types of autophagy,
which vary from each other based on the inducing signals and temporal aspects of the stimulation, type of cargo and mechanism of sequestration (101). The principal types of autophagy are macroautophagy, microautophagy and chaperone-mediated autophagy (101). The predominant form, macroautophagy (hereafter autophagy), is characterized by large double-membrane vesicles called autophagosomes that sequester and transport cytoplasmic material into lysosomes (102). This latter step is achieved through fusion of outer membrane of autophagosomes with lysosomes and subsequent luminal delivery of inner vesicle, which is degraded together with its cargo by lysosomal enzymes. The substrates of autophagic degradation include cellular organelles, protein aggregates, specific proteins, and even invading pathogens (103). The selective turnover of organelles by autophagy is defined with different names, e.g. the term mitophagy is used for mitochondria, reticulophagy for ER and lipophagy for lipid droplets (104).

Autophagy is a membrane-dependent process that is initiated by the formation of a pre-autophagosomal structure or phagophore assembly site (PAS), which leads to the generation of a cistern called an isolation membrane or phagophore. With the discovery of autophagy-related genes (ATG) in yeast and subsequent in-depth studies of their homologues in various cellular and animal models, many additional physiological processes have been linked to autophagy including intracellular quality control, maintenance of cellular and tissue homeostasis, anti-aging processes, cell differentiation and development, and innate and adaptive immunity (102).

Autophagosome biogenesis and fusion entails a series of discrete steps, i.e. initiation, phagophore nucleation, phagophore elongation and completion, and autophagosome fusion with lysosomes. Autophagosome biogenesis is initiated through the regulation of UNC-51-like kinase (ULK) complex, which is composed of ULK1/2 kinase, ATG13, FIP200 and ATG101 (Fig. 3, step 1). Multiple signaling cascades regulate the ULK complex activity. The best characterized one is the one centered around the mammalian target of rapamycin complex 1 (mTORC1). In presence of nutrients, active mTORC1 inhibits the ULK complex by phosphorylating ULK1/2 and ATG13 (105). Inactivation of mTORC1 by low levels of amino acids leads to dephosphorylation, translocation and activation of the ULK complex from cytosol to so-called PAS, the functional site where an autophagosome will be generated. There, it recruits other elements of the ATG machinery, such as autophagy-specific class III phosphatidylinositol 3-kinase (PtdIns3k) complex (105) (Fig. 3, step 2). This complex is formed by VPS34, p150, BECLIN1/ATG6 and ATG14L, and interacts with various factors
such as AMBRA1 and transmembrane VMP1, which regulate its localization and activation. The generation of phosphatidylinositol-3-phosphate (PtdIns3P) by PtdIns3k complex at PAS triggers the recruitment of proteins binding to this lipid, including WIPI proteins and DFCP1, which also participate in the biogenesis of phagophore (106). Another protein essential for the initial steps of autophagosome formation is the transmembrane protein ATG9A. This protein is principally distributed to trans-Golgi network and late endosomes, and dynamically associates with PAS (107, 108). Once formed, phagophores are elongated and closed, and these events require two ubiquitin-like molecules, ATG12 and the ATG8/LC3 proteins, which are part of two different but interconnected conjugation systems (Fig. 3, step 3). In the first ubiquitin conjugation-like reaction, ATG12 covalently linked to ATG5 through the action of E1-like and E2-like enzymes ATG7 and ATG10, respectively, before associating to ATG16L1 and forming ATG12-ATG5/ATG16L1 complex (105). This complex is essential for autophagosome biogenesis by targeting the second ubiquitin-like conjugation system to PAS and promoting its last step. The latter involves conjugation of phosphatidylethanolamine (PE) to the members of ATG8/LC3 protein family, which is subdivided in LC3 subfamily (composed in human by LC3A, LC3B and LC3C) and GABARAP (composed by human GABARAP, GABARAPL1 and GATE-16) subfamilies (109). After their synthesis, these ubiquitin-like proteins are post-translationally processed at C-terminus by ATG4 proteases to generate so-called non-lipidated LC3-I form (103). Upon autophagy induction, LC3-I becomes conjugated to PE at its C-terminus on both inner and outer membrane of the growing phagophore through action of ATG7 and E2-like enzyme ATG3 (103). The lipidated form of ATG8/LC3 proteins is known as LC3-II.

It has recently been shown that autophagy can target specific cargo, such as aggregated proteins, damaged or superfluous organelle, and invading pathogens. These selective types of autophagy involve a family of proteins called autophagy receptors, which are characterized by the ability to recognize degradation signals on cargo proteins and also bind members of ATG8/LC3 protein family on inner surface of the forming autophagosome (110). Different autophagy receptors recognize one or more distinct cargos and depending on the nature of cargo, several selective types of autophagy have been described: protein aggregates (aggrephagy), mitochondria (mitophagy), ribosomes (ribophagy), pathogens (xenophagy), peroxisomes (pexophagy), ER (reticulophagy), nuclear envelope (nucleophagy), liposomes (lipophagy), lysosomes (lysophagy)… (111). Some of the known soluble autophagy receptors, including p62/SQSTM1, optineurin
(OPTN), neighbor of BRCA1 (NBR1), and nuclear dot protein 52 kDa (NDP52), harbor both an ubiquitin-binding domain and a LC3-interacting region (LIR) (112). Thus, the unique structure and biochemical activity of autophagy receptors determine cargo recognition as they allow autophagosomes to specifically and exclusively sequester the structures targeted to degradation(113).

Generally, complete autophagosomes fuse with lysosomes to form autolysosomes (105) (Fig 3, step 4). This membrane fusion is mediated by a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (114, 115), which include VAMP8, SNAP25, SNAP29 and SYNTAXIN17 (STX17). STX17 has a unique C-terminal hairpin structure mediated by two tandem transmembrane domains containing glycine zipper-like motifs, which is essential for its association with outer membrane of completed autophagosomes once those are formed and guide their specific fusion with late endosomes/lysosomes (116). Together with SNARE complex, fusion of autophagosomes with lysosomes, similarly to other transport routes to the same destination, requires coordinated action of Rab GTPases and HOPS tethering complexes (117-119). Rab GTPases have a central role in fusion cascades. They are held soluble in the cytosol by GDP-dissociation inhibitor (GDI) proteins, which bind GDP-loaded Rab proteins. Once on membranes, a Rab-specific guanine nucleotide exchange factor (GEF) converts Rab GTPases into their active GTP-form (118), promoting their interaction with effectors such as tethering factors (119). The Rab7 GTPase is required for the fusion of endosomes with lysosomes and lysosomal transport along microtubules (120). In yeast, the Rab7-homolog Ypt7 binds to HOPS tethering complex, which in turn supports SNARE assembly and fusion. Rab7 as well as Ypt7 are also required for fusion of autophagosomes with endosomes (121-123) and detected on autophagosomes (124). However, how autophagosomes become fusion competent with lysosomes is still poorly understood.

During fusion of autophagosomes with lysosomes, the LC3-II pool present in the interior of autophagosomes is also delivered in lysosomal lumen and therefore is very often used as a marker protein to monitor the entire autophagy pathway. Autophagosomal cargos are finally degraded by lysosomal enzymes, which include proteases, lipases, nucleases and glycosidases (125). Breakdown products, i.e. amino acids, lipids, nucleotides and carbohydrates, are released into cytosol by lysosomal surface transport and permeases, and reused in biosynthetic and metabolic pathways (102).
Figure 3. Schematic view of macroautophagy in mammalian cells. Upon autophagy induction, the ULK complex, which consists of ULK1, ATG13, FIP200 and ATG101, is central in triggering the formation of the phagophore assembly site (PAS) (step 1). mTOR, which senses levels of amino acids, glucose and growth factors, as well as specific genotoxic and ER stress, is one of the principal signaling hub controlling autophagy. Following stimulatory signals, mTOR is inactivated and the ULK complex becomes hypophosphorylated. Nucleation of the phagophore also requires ATG9A and the class III PtdIns3k complex, which includes the VPS34 lipid kinase and its regulatory subunits ATG14L, VPS15 and BECLIN1 (step 2). The elongation of the phagophore membrane and the completion of autophagosomes involve two ubiquitin-like conjugation pathways (step 3). The first produces the ATG12-ATG5 conjugate, which forms a multimeric complex with ATG16L, whereas the second results in the conjugation of LC3 to phosphatidylethanolamine (PE). LC3-PE is required for the expansion of phagophore, specific recognition of the autophagic cargoes and fusion of autophagosomes with lysosomes. Autophagosomes finally fuse with endocytic and lysosomal compartments, ultimately leading to formation of the autolysosome (step 4).
3.2 Unconventional role of autophagy-related proteins

It has been believed for a long time that ATG proteins were exclusively involved in autophagosome formation. A series of discoveries, however, have revealed that they are also participating individually or in functional groups in pathways distinct from autophagy (126). The growing appreciation for other functions of ATG proteins is similar to the explosion of research in ubiquitin field, which began with a narrow focus on the role of ubiquitination in protein turnover, but has now broadened to study its tremendous impact on many aspects of cellular physiology, including protein sorting, DNA repair, gene regulation, protein retrotranslocation, apoptosis and the immune response (126, 127).

Studies on binding partners of the members of the LC3 protein family have revealed many new interacting components, including several that are in cellular pathways other than autophagy. LC3 proteins bind to their interactors very often through LIR motifs (104, 111, 128). Furthermore, several cryptic LIR sequences are activated by phosphorylation of serine and threonine residues in their vicinity or formed through rearrangements of protein conformation (129), thereby expanding the number of putative LC3 protein interacting factors. Among the non-autophagy-related proteins that interact with LC3 proteins are GTPases (130), GTPase-activating proteins (131, 132) and guanine-nucleotide exchange factors (133). Although these are not direct targets of the autophagic process, they might serve to regulate events involved in cellular response to autophagy or other cellular function when LC3 is targeted through conjugation to PE to other organelles such as endosomes (132-136). Another example for a non-autophagic role of LC3 is in the context of virus infections. As explained in detail in chapter 2 of this thesis, CoV trigger the formation of DMVs to generate their replication and transcription platforms. MHV appears to usurp host cell machinery for coat protein complex II (COPII)-independent vesicular transport route known as ER-associated degradation (ERAD) tuning pathway, which transports ER degradation-enhancing α-mannosidase-like 1 (EDEM1) and osteosarcoma amplified 9 (OS9) directly for ER to endolysosomal system, to produce its DMVs. As a result, EDEM1 and OS9 accumulated in these structures, which are also decorated with non-lipidated LC3-I. Knocking down of LC3 proteins, but not the inactivation of host cell autophagy, inhibits MHV infection (137). Equine arteritis virus (EAV) and Japanese encephalitis virus (JEV) also hijack the same ER-derived membranes to ensure their efficient replication (138, 139).

Several components of modulators of two ubiquitination-like systems of autophagy, singularly or in functional groups, are crucial for a variety of cellular
processes. In neuroendocrine PC12 cells, ATG16L1 localizes to hormone-containing dense-core vesicles through interaction with RAB33A (140). Although blocking autophagy with wortmannin is ineffective, knocking down of ATG16L1 causes a marked reduction of hormone secretion level (140). Moreover, two studies showed that ATG16L1 is also required for granule exocytosis pathway in intestinal Paneth cells (141, 142). Interestingly, non-autophagic roles of ATG proteins have often been unveiled by studying pathogens cell invasions. The infection of mammalian cells by many RNA viruses produces virus-derived immunostimulatory RNA (isRNA) structures. In particular, 5′-triphosphorylated RNA produced during virus life cycle is directly recognized by a cytoplasmic RNA helicase protein, RIGI, or generates dsRNA, which is recognized by an alternative RNA helicase, MDA5. These helicases have N-terminal caspase recruitment domains (CARDs) and interact with other CARD-containing proteins (143, 144). The ATG12-ATG5 conjugate negatively regulates isRNA-generated type I IFN production and interacts with the RNA helicases RIGI and MDS5, thereby interfering with the ability of these helicases in engaging in their normal CARD interactions and consequently inhibiting type I IFN production (143, 144). Furthermore, Hwang and co-workers have shown that ATG12-ATG5/ATG16L1 complex is required for IFN γ-mediated host defense against murine norovirus replication (145). In contrast, during hepatitis C (HepC) virus infection, another subset of autophagy proteins, specifically BECLIN1, LC3, ATG4B, ATG5, ATG7 and ATG12, are crucial for the translation of HepC RNA, viral replication and egress from cells, rather than interfering with the viral life cycle (146). Recently, Mauthe and colleagues found an undocumented role for ATG13 and FIP200 in picornavirus replication that is independent of their function in autophagy as part of the ULK complex (147).

In this chapter, I just highlighted some examples of non-autophagy function of ATG proteins. It is now evident that the ATG machinery is far more versatile in coordinating cellular activities than was previously appreciated, something that has recently been underlined with a ATG proteome-specific siRNA-based screen exploiting different viral infections to study the conventional or unconventional role of ATG proteins in promoting or suppressing infections (147, 148).

3.3 Autophagy and Coronavirus

The replication of CoV also takes place on DMVs, which are reminiscent of autophagosomes. Studies addressing whether these two structures were the same, revealed that this was not the case. Nonetheless, components of ATG machinery...
have been associated to CoV infections and these relationships are discussed in Chapter II.

3.4 Autophagy and Influenza A virus

The first study on the interaction between autophagy and IAV was carried out with A/WSN/33 (H1N1) and A/chicken/Beijing/04 (H9N2) strains (149). Infected cells displayed the presence of more autophagosomes, an increased lipidation of LC3 and enhancement of the autophagic flux, which indicated an induction of autophagy. Inhibition of autophagy with either 3-methyladenine, wortmannin or knockdown of BECLIN1 and LC3, markedly decreased both viral protein production and titers of IAV (149). Noticeably, the compounds utilized had no marked effect on virus entry or cell viability, either of which might limit viral replication (149). The induction of LC3 conversion by IAV infection has been confirmed in a number of cell lines by other groups as well (150, 151). Gannage and colleagues found that infection of human lung epithelial cells with IAV H3N2 and H1N1 strains prevents autophagosomes from fusing with lysosomes, with viral M2 protein being necessary and sufficient for this inhibition (150). This block leads to a perinuclear accumulation of autophagosomes (150). IAV M2 has a proton channel activity that during virus cell entry, triggers virion disassembly in response to lower endosomal pH, while its cytoplasmic tail contributes to virus particle assembly, budding, and morphogenesis (93). Treatment with amantadine, an IAV M2 proton channel inhibitor, was unable to prevent autophagosome accumulation in M2-transfected and H3N2- or H1N1-infected cells indicating that M2 proton channel activity is not involved in blocking autophagosome fusion with lysosomes (150). A different study, however, obtained contrasting results because upon treatment with amantadine, an inhibition of autophagosome accumulation was observed in H3N2-infected cells (152). Moreover, autophagosomes were not observed accumulating in cells exposed to H1N1 in the same investigation. Overall, these data indicate that the activity of the M2 proton channel plays a role only in blocking the fusion of autophagosomes with lysosomes, which might be a key mechanism used by the H3N2 strain to arrest autophagy (152). This debating data still needs to be further clarified. Randow and co-workers identified a LIR motif in M2, at amino acid positions 91-94, which directs transfected GFP-LC3 to plasma membrane without influencing perinuclearly accumulated GFP-LC3 puncta (153). The authors speculated that IAV subverts ATG components by mimicking a host protein-protein mechanism and this strategy may facilitate transmission of infection between organisms by enhancing the stability of virion
progeny. This was tested by infecting A549 cells with an IAV strain carrying M2 either WT or with a mutated LIR motif, and then check colony formation by plaque assay using the released virus immediately or left at room temperature for 1-2 days. The virions of the strain expressing the M2 LIR motif mutant lost infectivity over time (153).

In a recent work, it has been shown that the WD repeat-containing C-terminal domain (WD40 CTD) of ATG16L1 is essential for non-canonical LC3 recruitment to endolysosomal membranes, but dispensable for canonical autophagy (154). Dendritic cells from mice expressing a form of ATG16L1 lacking the WD40 CTD did not display a re-distribution of GFP-LC3 to the plasma membrane when were infected with IAV, which also indicated that LC3 lipidation is part of a non-canonical autophagy pathway (154). However, the impairment of this program did not affect viral titers (154). Future studies are needed to elucidate the function and relevance of this non-canonical autophagy pathway during the IAV infection cycle.

IAV is a major human pathogen for which there are few treatment options. To search for novel potential therapeutic targets while systematically investigating virus-host interaction, various comprehensive proteomics and genome-wide screens have been performed in IAV-infected cells (155-157). In two of the screens, mTORC1 was identified as a protein complex that promotes IAV infection (158, 159), and consistently, some groups have found that H1N1 IAV activates mTORC1 (159, 160). mTORC1 is central in modulating the metabolism of the cell through regulation of a plethora of cellular processes, including autophagy (161). Conversely, IAV replication is reduced when mTOR is inhibited directly (158) or indirectly, by chemically blocking REDD1, a known negative regulator of mTORC1 (159). The role of mTORC2 during IAV infection, in contrast, remains unknown. A recent paper, however, found that mTORC2 and PDPK1 differentially phosphorylate AKT upon IAV infection and the viral NS1 protein promotes phosphorylation of AKT at a different site via mTORC2, which is an activity dispensable for mTORC1 stimulation (162), but known to regulate apoptosis (163). Depletion of ATG5 or ATG7, however, did not affect mTORC1 activation over the course of IAV infection (162). Altogether, these data suggest that mTORC1 activation supports IAV protein expression and replication, but not through autophagy.

4. Outline of this thesis

Chapter II. The interaction between Nidovirales and autophagy components
In this chapter, we reviewed the current knowledge about the interplay between Nidovirales and autophagy, as numerous viruses belonging to this order trigger autophagy in host cells. Nidovirales are subdivided into four virus families: Roniviridae, Arterividae, Mesoniviridae, and Coronaviridae. Although the interaction between autophagy and viruses from some of these virus families have been extensively studied, it remains to be established whether there is an interaction between autophagy and Torovirinae, Mesoviridae and Roniviridae. Moreover, for specific viruses like porcine reproductive and respiratory syndrome virus (PRRSV) and TGEV, opposite conclusions have been reported regarding whether autophagy induction is beneficial or detrimental for viral life cycle and these effects should be clarified in further studies. This chapter also provides a critical compendium of the available data.

Chapter III. Ultrastructural characterization of membrane rearrangements induced by porcine epidemic diarrhea virus infection

The intracellular membrane reorganization induced by CoV has been investigated during the infection of numerous viruses belonging to this virus family, but never with a member of α-CoV subfamily. In this chapter, we qualitatively and quantitatively examined at ultrastructural level that the membrane rearrangements over the course of the infection of the porcine epidemic diarrhea virus (PEDV), an α-CoV, using electron microscopy. We observed that PEDV initially induces the formation of DMVs and CMs, which probably serve as replication and transcription platforms. We also found that viral particles start to form almost simultaneously in both ER and in large virion-containing vacuoles (LVCVs), which are compartments originating from Golgi. Importantly, our results indicate together with Golgi, ER is the major platform of virion assembly and this distinguishes α-CoV from viruses belonging to other CoV subfamilies.

Chapter IV. Coronavirus nucelocapsid proteins assemble constitutively in high molecular oligomers

Our goal in this chapter was to clarify the mechanism and relevance underlying the N protein oligomerization in CoV life cycle. We first confirmed in vitro that recombinant MHV and SARS-CoV N protein self-interacts and form large oligomers. Analysis of the interaction between different N protein truncations revealed at least three regions of N proteins are able to cross-interact between each other in an interchangeable manner, providing a possible mechanism for
Chapter I

oligomerization. We also found that MHV N protein forms oligomers in cytoplasm upon synthesis and its oligomerization does not require binding to gRNA. Thus, we hypothesized that N protein oligomers provide a larger binding surface for the gRNA, which will be thus optimally entwined at the site of replication to be subsequently incorporated into viral particles.

Chapter V. The recruitment of mouse hepatitis virus (MHV) nucleocapsid protein to the replication-transcription complexes plays a key role in infection

In addition to being part of viral particles, CoV N protein also localizes to RTCs. The goal of this chapter was to shed light into the relevance of this localization for CoV life cycle. We found that nsp3 is the only non-structural protein of RTCs that interacts with CoV N protein. We have then investigated the importance of this interaction during CoV infection by identifying and studying the domains of MHV N protein involved in its association with nsp3. We found that specific amino acids in N1b and N2a regions of the N protein mediate its interaction with nsp3. N protein variants carrying point mutations in these critical amino acids fail to be recruited to RTCs and have a dominant negative effect on MHV infection by impairing virus replication and progeny production. We also showed that gRNA binding to N oligomers is not essential for N-nsp3 interaction but nonetheless hypothesized that recruitment of N protein to RTCs could favor the localization of gRNA at this site early in infection, possibly also promoting viral transcription and replication.

Chapter VI. Autophagy-independent role of specific ATG8 homologs in influenza virus infection

In this chapter, our goal was to better understand the interaction between IAV and ATG machinery. We found that IAV infection triggers autophagy, but an intact ATG machinery is not required for IAV infection. Interestingly, a subset of ATG8 proteins, however, associate with the cytoplasmic vRNPs in their non-lipidated form. Crucially, members of LC3 protein subfamily, but not those belonging to the GABARAP protein subfamily, play a pivotal role in IAV infection. Thus, our data have revealed a similar unconventional relationship between IAV and CoV, with LC3 protein subfamily, although the role of non-lipidated LC3 proteins in the life cycle of these two viruses might be different.
Chapter VII. Summary and perspectives

CoV and IAV are two distinct families of viruses, but both can cause severe acute respiratory infections and represent serious threats to global health. In this chapter, we first summarized the intracellular membrane rearrangements induced by CoV and the role of N protein during life cycle of MHV. Moreover, we also discuss the available literature on the relationship between IAV and autophagy in the context of our discoveries, and propose that the unconventional roles of LC3 may not only important for CoV infections, but also those of IAV.
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