Non-Lipidated LC3 is Essential for Mouse Hepatitis Virus Infection

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Abstract

Coronaviruses (CoVs) are enveloped viruses responsible for severe respiratory diseases in birds and mammals. In infected cells they induce double-membrane vesicles (DMVs) and convoluted membranes (CMs), which are thought to be the site of virus replication. Until recently, both the origin of the CoV-induced vesicles and the exact localization of CoV replication remained unknown. It was assumed that the vesicles derive from the endoplasmic reticulum (ER). Nevertheless no conventional protein markers of the ER, ER-to-Golgi intermediate compartment (ERGIC), Golgi, or coatomer proteins could be detected in these structures.

Recent data from our laboratory and others shed light on this mystery. It appears that the Mouse Hepatitis Virus (MHV), a prototype CoV, co-opts ERAD tuning vesicles as replication platforms. These

vesicles are released from the ER, but do not contain conventional ER markers or coatomer proteins. Rather, they contain ERAD factors such as SEL1L, EDEM1, and OS-9 that are constitutively cleared from the folding compartment by so called ERAD tuning programs, and display non-lipidated LC3 (LC3-I) periferically associated at their limiting membrane. In MHV-infected cells, the ERAD tuning vesicle markers co-localize with viral non-structural proteins and double-stranded RNA, which are DMV markers. The unconventional role of LC3-I in the MHV infection cycle is further supported by the fact that Atg5 and Atg7, both essential proteins for LC3-I to LC3-II conversion and macroautophagy, are dispensable for CoV replication and DMV formation. These new insights into CoV replication might lead to new therapies to treat CoV infections. They also reveal a novel role for LC3, in its non-lipidated form, in both maintenance of cellular proteostasis and viral infection, the latter function supported by recent findings showing involvement of LC3-I in equine arteritis virus replication.
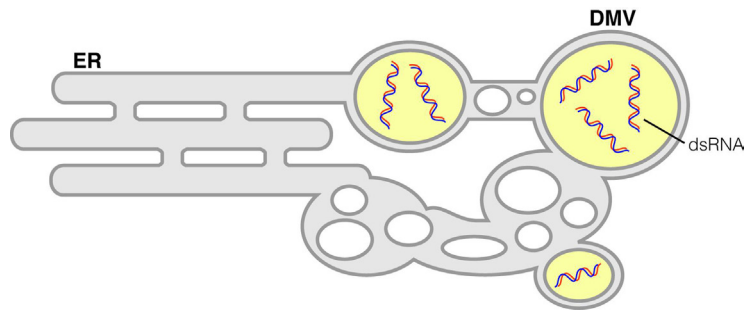
CORONAVIRUSES

Coronaviruses are enveloped positive-stranded RNA viruses belonging to the family Coronaviridae. They cause diverse diseases in avians and mammals, usually with a mild outcome, but can also lead to severe respiratory infections, diarrhea, or neurological diseases in humans. One prominent example is the severe acute respiratory syndrome (SARS) CoV, which led to the death of several hundred people worldwide during the outbreak in 2002/2003. CoVs are transmitted via the respiratory route through aerosols, or via the fecal–oral route.

Coronavirus Replication

Coronavirus virions have a diameter of 80–120 nm and contain the largest known RNA genome (~30 kb of positive-sense single-stranded RNA). Three viral structural proteins, membrane (M), envelope (E), and spike (S), are anchored in the host-derived viral envelope, while the nucleocapsid (N) protein encloses the RNA genome. The S protein mediates the first steps of virus entry, namely the binding to the respective host–cell receptor, during which it undergoes conformational changes, and the subsequent fusion with the host cell membrane. The S protein variants determine host cell tropism. Entry of the virion into the cell is followed by its disassembly and the release of the genomic RNA into the cytoplasm, where it is directly translated into the viral replicase polyproteins pp1a and pp1b. A viral-encoded proteinase then cleaves the polypeptides into 16 non-structural proteins (nsps). One of these is the viral RNA polymerase, which uses the positive RNA strand to synthesize the negative strand, which in turn serves as a template for the synthesis of subgenomic mRNAs encoding for structural proteins (S, E, M, N). Other nsps are thought to induce membrane rearrangements in the host cell that lead to the formation of double-membrane vesicles (DMVs) and convoluted membranes, giving rise to a reticulovesicular network (Figure 8.1). This network is associated with the viral replication–transcription complexes (RTCs) (Gosert et al., 2002; Snijder et al., 2006), thereby providing a replication platform, which has several advantages for the virus: (1) the viral genome is protected from the host cell's degradative enzymes; (2) the dsRNA intermediate produced during replication is isolated and therefore cannot trigger the host cell's immune response (Versteeg et al.,

FIGURE 8.1 Coronavirus-infected cells show dramatic membrane modifications of the ER that form a reticulovesicular network including DMV structures. Viral replication factors were found in reticular structures close to the DMVs, while dsRNA was mainly found in DMVs without connection to the cytosol, which leaves open the question of how the newly synthesized RNA finds its way to the places of virus assembly.

2007; Zhou and Perlman, 2007); and (3) membrane lipids of the host cell membranes can be used for the viral envelope. After successful replication, the genomic RNA and the N proteins form the nucleocapsid. Although other structural proteins are found in different subcellular locations (M protein in the Golgi apparatus, E protein in the ER, and S protein in the secretory pathway and at the plasma membrane), the virions assemble through ER–Golgi intermediate compartment (ERGIC) membrane invagination and budding events into the ERGIC. From the lumen of the ERGIC, the virions are released from the cell via the secretory pathway.

Double-Membrane Vesicle Origin

For a long time, the origin of the CoV-induced DMVs has been a matter of dispute. It was first assumed that components of the cellular autophagic machinery are needed for formation of CoV-induced DMVs. In fact, Prentice et al. (2004) found that the DMVs contain autophagy components, and that CoV replication in embryonic stem cells is dependent on Atg5 – an essential autophagy gene. However, later studies by Zhao et al. (2007), as well as studies performed in our laboratory (Reggiori et al., 2010), revealed that the CoV MHV can also replicate in cells lacking Atg5 or Atg7 autophagy genes. These studies demonstrate the dispensability of an intact autophagic machinery for CoV infection, DMV formation, viral replication, and generation of the viral progeny.

Ultrastructural analysis established the ER as the most likely source of CoV-induced DMVs (Snijder et al., 2006; Knoops et al., 2008). Indeed, infected cells show dramatic membrane modifications of the ER, with formation of a reticulovesicular network including DMV structures (Figure 8.1) that later in infection convert into huge, single-membrane vesicles (Knoops et al., 2008). Viral replication factors were found in reticular structures close to the DMVs, while dsRNA was mainly found in DMVs without connection to the cytosol, which leaves open the question of how the newly synthesized RNA finds its way to the places of virus assembly. Further evidence of the ER origin of DMVs came from analyses of nsp localization and function. nsp4 and nsp6 were found to be N-glycosylated (a feature of

proteins processed in the ER), to be localized in the ER membrane, and to induce DMV-like structures when ectopically expressed in cells (Gadlage *et al.*, 2009).

MICROTUBULE-ASSOCIATED PROTEIN 1 LIGHT CHAIN 3 (LC3)

Microtubule-associated protein 1 light chain 3 (MAP1LC3, hereafter referred to as LC3) is an 18-kDa protein that was first described as a component of the microtubule-stabilizing complexes comprising MAP1A and 1B in neurons (Mann and Hammarback, 1994). Besides stabilizing microtubules, MAP1A and MAP1B were shown to act as adaptor proteins in complexes regulating neuronal development or signaling (reviewed in Halpain and Dehmelt, 2006). The fact that MAP1A and MAP1B are mainly expressed in neuronal cells, while LC3 is also expressed in other tissues, clearly speaks for additional roles of LC3. Some years later, Kabeya *et al.* (2000) reported that LC3 is a homologue of Atg8, an essential gene for autophagy in yeast, and investigated its involvement in mammalian autophagy. Two forms of LC3 were described: LC3-I, which is found in the cytosol, and LC3-II, which is covalently associated to the autophagosome limiting membrane (Kabeya *et al.*, 2000). LC3-II has been used as a marker for autophagosomes ever since.

LC3 Conversion

The precursor protein proLC3 is co-translationally cleaved at its C-terminus by the cysteine protease Atg4B/autophagin1. This leads to the formation of LC3-I, which exposes a carboxy terminal glycine at position 120 (Figure 8.2). Activation of autophagy leads to a series of sequential reactions consisting in the activation of LC3-I by the E1-like enzyme Atg7, and the intervention of the E2-like enzyme Atg3 and of the Atg5–Atg12 complex that, acting as an E3 ligase, conjugates LC3-I to phosphatidylethanolamine (PE) at the pre-autophagosomal membrane generating the LC3-II form (reviewed in Tanida, 2010) (Figure 8.2). While the autophagic cargo (i.e., cytoplasmic components, damaged organelles) is engulfed, the pre-autophagosome matures into an autophagosome and increasing
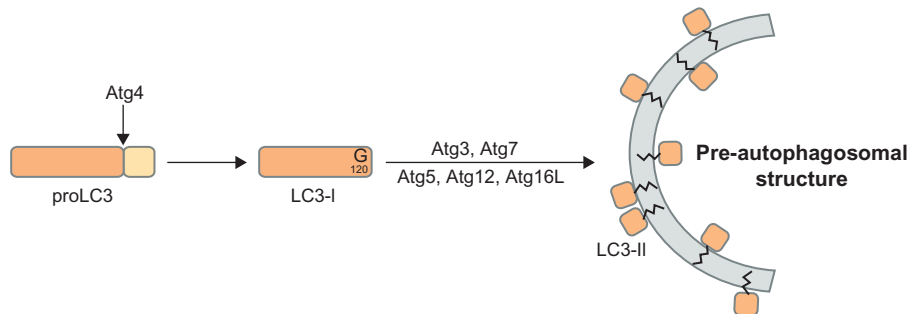
FIGURE 8.2   LC3 is synthesized as a precursor protein, proLC3, which is co-translationally cleaved at its C terminus by the cysteine protease Atg4B/autophagin1. This leads to the formation of LC3-I, which exposes a carboxyterminal glycine at position 120 (G120). LC3-I is activated by the E1-like enzyme Atg7, and transferred to the E2-like enzyme Atg3. The Atg5–Atg12–Atg16L complex, which acts as an E3 ligase, conjugates LC3-I to phosphatidylethanolamine (PE) at the membrane of the pre-autophagosomal membrane, resulting in LC3-II.

amounts of LC3-II are inserted into both its inner and outer membrane. After autophagosome–lysosome fusion into the autolysosome, the LC3-II on the cytosolic face of the autophagosomal membrane is recycled back to LC3-I by Atg4B-mediated de-lipidation. On the other hand, the LC3-II located in the intra-autophagosomal membrane is degraded by lysosomal enzymes together with the autophagosomal cargo. The role of LC3 in autophagy is probably the mediation of microtubule plus-end-directed transport of the autophagosome to the lysosome/late endosomes via binding to the Rab7 effector FYCO1 (Pankiv *et al.*, 2010).

LC3-I and Endoplasmic Reticulum-Associated Degradation Tuning

About one-third of the human proteome is synthesized by ribosomes attached at the cytosolic face of the ER membrane. A multitude of ER-resident molecular chaperones and folding factors assist in the folding and the maturation of nascent polypeptide chains that enter the ER lumen co-translationally. Once the native structure is attained, the proteins leave the ER and are transported through the secretory pathway to their final intra- or extracellular destinations. Protein quality control machineries ensure that only properly folded and assembled proteins leave the ER.

Polypeptides that do not attain the native structure in due time must be removed from the ER and degraded. A complex series of events, collectively called ER-associated degradation (ERAD), targets misfolded polypeptides for dislocation into the cytosol, polyubiquitination, and subsequent degradation by the cytosolic 26S proteasomes. Maintenance of cellular proteostasis relies on tight regulation of the ER-folding, quality-control, and degradation machineries. Pathologic or experimental conditions that enhance ERAD activity may cause premature interruption of ongoing folding programs. As a consequence, polypeptides that have not yet attained the native structure might be selected for disposal. On the other hand, insufficient ERAD activity may cause intracellular accumulation of misfolded conformers and may impair ER quality control, thus leading to secretion of misfolded polypeptides that should rather be retained intracellularly and degraded. Accumulation of high levels of misfolded proteins in the ER leads to induction of the unfolded protein response (UPR), resulting in transcriptional upregulation of chaperones, which can take several hours to occur. It has recently been proposed that several mechanisms operate in mammalian cells to more rapidly adapt the content and the activity of select ER-resident factors to fluctuations in ER cargo load (Calì *et al.*, 2008; Bernasconi *et al.*, 2012; Chambers *et al.*, 2012). For example, ERAD activity is tightly controlled by post-translational mechanisms, collectively termed ERAD tuning. These mechanisms regulate the turnover of select ERAD factors, their subcellular localization, and their inclusion in functional supramolecular complexes, depending on the level of misfolded proteins in the ER. In recent publications, we have shown that ERAD factors such as EDEM1 and OS-9 are constitutively segregated from the ER into so-called ERAD tuning vesicles (Figure 8.3). This selective segregation does not involve any component of the conventional secretory pathway. Instead, the ER membrane-anchored protein SEL1L and the cytosolic LC3-I act as an ERAD tuning receptor to selectively deliver OS-9 and EDEM1 into the ERAD tuning vesicle. Detailed studies have revealed that LC3-I is non-covalently associated to the vesicle membrane, and that it associates to the cytosolic tail of SEL1L or of a SEL1L-associated protein (Cali *et al.*, 2008; Bernasconi *et al.*, 2012).
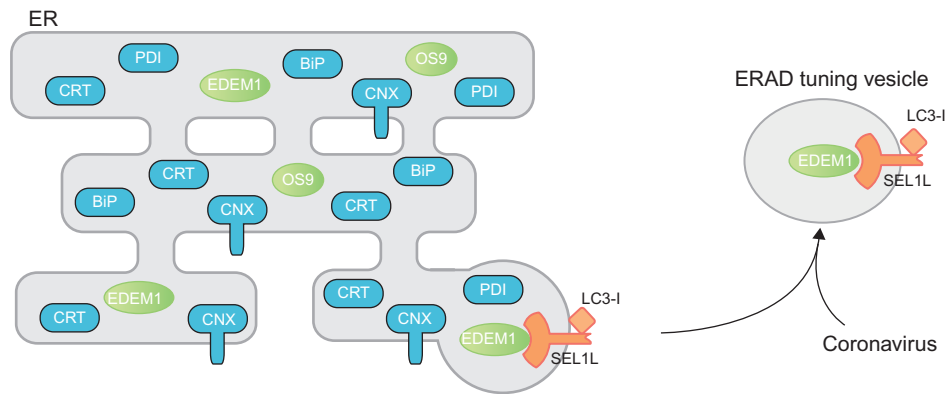
FIGURE 8.3   **Model for the vesicle-mediated segregation of EDEM1 and OS-9 from the mammalian ER.** At steady state, the SEL1L LC3-I complex acts as an ERAD tuning receptor that delivers the ERAD factors EDEM1 and OS-9 from the ER lumen into ERAD tuning vesicles. Conventional ER chaperones, here depicted in blue, remain in the ER. Note that it is still unclear whether LC3-I associates directly or indirectly with SEL1L. CNX, calnexin; CRT, calreticulin; BiP, 78-kDa glucose regulated protein; PDI, protein disulfide isomerase.

UNCONVENTIONAL ROLE OF NON-LIPIDATED LC3 IN MHV REPLICATION

One of the crucial events during the MHV infection cycle is the formation of DMVs, where the membrane-associated RTCs ensure replication of the viral genome. DMVs are part of a reticulovesicular network of modified ER membrane, but the precise membrane lipid origin and protein content has long been unclear. The involvement of autophagy genes and, hence, of LC3 in the formation of DMVs and in MHV replication, has also been a matter of debate.

A collaboration between our group and the groups of Reggiori and de Haan revealed that the MHV proteins nsp2/3 and viral dsRNA co-localize with the known ERAD tuning vesicle markers EDEM1, OS-9, SEL1L and LC3-I, but not with the autophagosome marker GFP-LC3-II (Reggiori *et al.*, 2010; Bernasconi *et al.*, 2012). Our studies also revealed that the autophagy gene Atg7, and hence the conversion of LC3-I to LC3-II, is dispensable for DMV formation (as it is for the formation of the ERAD tuning vesicles) and for viral replication. Significantly, siRNA-mediated silencing of LC3 or SEL1L expression inhibits MHV replication and virion production, a phenotype that is efficiently reverted upon back-transfection of a G120R LC3 mutant that cannot be lipidated. These data show that LC3-I and SEL1L are required and sufficient to support MHV replication. These findings strongly suggest that MHV might co-opt the cellular ERAD tuning vesicles, containing EDEM1, OS-9, and SEL1L, and decorated with LC3-I, as platforms for the replication of the viral genome.

More recently, similar observations were made for the equine arteritis virus (EAV). This virus also induces dsRNA-containing DMVs that are positive for EDEM1 and display LC3-I at their limiting membrane. Moreover, EAV does not require a functional autophagic machinery for infection, and possibly hijacks the same ERAD tuning pathway as MHV (Monastyrska *et al.*, 2013).

DISCUSSION

The intracellular level of a given protein might be regulated at the transcriptional, translational, or post-translational level. While the former two mechanisms are rather slow responses, since they involve mRNA transcription and/or protein synthesis, respectively, the latter is more rapid and readily reversible. The work of our group and others established an unanticipated role of non-lipidated LC3 in a post-translational mechanism, called ERAD tuning, that regulates constitutive ERAD activity and the adaptation of ERAD activity to fluctuations in ER cargo load with misfolded polypeptides. By binding to the cytosolic tail of the ER membrane protein SEL1L (or of a SEL1L-associated protein), LC3-I participates in an ERAD tuning receptor that removes ERAD factors such as OS-9 and EDEM1 from the ER lumen and delivers them in cytosolic ERAD tuning vesicles. ERAD factors' segregation from the ER is one way to set ERAD activity at levels that do not interfere with ongoing folding processes in the ER (Cali *et al.*, 2008; Bernasconi *et al.*, 2012). In this way, cells guard against hyper-ERAD activity, which can result in loss-of-function phenotypes. Examples thereof are increased sarcoma metastasis (Tsai *et al.*, 2007) and rheumatoid arthritis (Yamasaki *et al.*, 2005; Joshi *et al.*, 2010), where elevated levels of specific E3 ubiquitin ligases result in the disease-causing inappropriate destruction of native proteins. As shown for many other intracellular pathways, the vesicle-mediated removal of ERAD factors from the ER is hijacked by pathogens. Viruses such as MHV and EAV co-opt the EDEM1- and OS-9-containing vesicles as platforms for their replication, and/or to protect the viral ssRNA from the detection by the host's immune system. Also in this context, non-lipidated LC3 plays a crucial role, as silencing of its expression (and silencing of SEL1L, the second component of the ERAD tuning receptor) inhibits viral replication. Efficient DMV formation and viral replication in cells lacking Atg5 or Atg7, both proteins being involved in LC3-I to LC3-II conversion, demonstrates that lipidation of LC3-I is not required for viral replication. This novel finding is surprising, since LC3-I was commonly regarded as the non-functional precursor of the lipidated autophagosome marker LC3-II. However, individual reports have indicated more diverse functions of this protein besides the autophagic pathway. For example, non-lipidated LC3 has been shown to mediate the association of caldendrin to the microtubule cytoskeleton (Seidenbecher *et al.*, 2004). Furthermore, in the organism *Pichia pastoris*, non-lipidated Atg8, the yeast homologue of LC3, plays a role in homotypic vacuole fusion (Tamura *et al.*, 2010). Inclusions of the bacterium *Chlamydia trachomatis* in human cells are connected to the microtubular network via non-lipidated LC3, while lipidated LC3 does not co-localize with the inclusions. Also, non-lipidated LC3, but not an intact autophagic machinery, is essential for the development of the pathogen (Al-Younes *et al.*, 2011). All these examples hint at unappreciated roles of LC3 in its cytosolic, non-lipidated form, alongside its crucial role in autophagy upon lipidation.

In the context of CoV infection, this finding might offer novel therapy approaches. It is not the autophagic machinery that should be targeted to stall CoV infection but, rather, more specifically, LC3 or other components of the ERAD tuning machinery (e.g., SEL1L). To date, the detailed mechanism of the formation of the ERAD tuning vesicles has not been described, and the identification of more essential ERAD tuning factors will provide additional potential drug targets.

Acknowledgments

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