Foot-and-mouth disease virus 3A hijacks Sar1 and Sec12 for ER remodeling in a COPII-independent manner

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Abstract

Positive-stranded RNA viruses modify host organelles to form replication organelles (ROs) for their replication. Enteroviral 3A protein has been demonstrated to be highly associated with the COPI pathway, in which factors work on the ER-to-Golgi intermediate and the Golgi. However, Sar1, a COPII factor exerting coordinated action at endoplasmic reticulum (ER) exit sites, rather than COPI factors, is required for foot-and-mouth disease virus (FMDV) replication. Therefore, we thought that deep understanding of FMDV 3A was the key to explaining the differences and to unlocking the secret of FMDV RO formation. In this study, FMDV 3A was confirmed as a peripheral membrane protein capable of modifying the ER into vesicle-like structures, which were neither COPII vesicles nor autophagosomes. When the C-terminus of 3A was truncated, it would be located at the ER without vesicular modification. This change was revealed by mGFP and APEX2 fusion constructs observed by fluorescence microscopy and electron tomography, respectively. Referring to other 3A truncation, the minimal region for modification was aa 42–92. Furthermore, we found that the remodeling was related to two COPII factors, Sar1 and Sec12. Both interacted with 3A, but their binding domains on 3A were different. Finally, we hypothesized that the N-terminus of 3A would interact with Sar1 as its C-terminus simultaneously interacted with Sec12, which possibly would enhance Sar1 activation. On the ER membrane, two active Sar1 were connected by 3A with regions of aa 42–59 and aa 76–92, causing curvature of the membrane. This mechanism is distinct from the traditional COPII pathway and should be crucial for FMDV RO formation.

Introduction

All positive-stranded RNA viruses, including enterovirus and FMDV (family *Picornaviridae*), modify the host organelle to achieve their own replication. The modified organelles are termed “replication organelles” (ROs)^{1,2}. It was postulated that ROs could serve as platforms to recruit key components for viral replication, concentrate resources in favor of the virion package^{3,4}, and shield viral RNA from RNase or RNA sensors⁵. Although the mechanism of RO formation differs in different virus genera and is still obscure, enterovirus (including poliovirus (PV), coxsackie B virus (CBV), and rhinovirus) could represent a paradigm. First, PV 3A protein hijacks COPI factor GBF1, resulting in the inhibition of Arf1 activation⁶ and secretory blockage⁷. However, the biological function should be verified. In a recent study, it was suggested that Arf1 activation directly supported RO formation⁸. In addition, 3A accumulation requires 3A-GBF1 interaction⁹. On the other hand, PV 3A also interacts with ACDB3, further recruiting PI4KB for phosphorylating PI (phosphoinositol) lipid into PI4P^{10,11}, which might attract viral 3D polymerase¹¹, 3C protease¹², and host protein OSBP¹³. OSBP further exchanges PI4P for cholesterol from the ER. Cholesterol accumulation mediates viral precursor protein processing¹³ and might be one of the factors in RO formation. Collectively, viral 3A (or 3AB) protein could represent an RO marker protein highly associated with enteroviral RO development^{13,14}. However, this has not been proven in FMDV. Numerous differences exist between FMDV and enterovirus. **1)** FMDV is resistant to GBF1 inhibitors^{15,16} and highly associated with the COPII pathway, rather than COPI¹⁶. **2)** Combination of FMDV 2B and 2C, instead of

3A, would block the secretory pathway in the TsO45 VSV G protein trafficking model^{17,18}. **3)** According to the results of transmission electron microscopy (TEM) from Monaghan *et al.*'s study, FMDV RO is homologous and gathers beside the nucleus, while enteroviral RO appears as heterologous vesicles or tubules^{2,19}. **4)** FMDV does not require PI4K or PI4P^{20,21}. **5)** The C terminus of FMDV 3A is longer than those of other picornaviruses and has an unknown biological function. Therefore, more efforts are required to unlock the secrets of FMDV RO formation, especially those of FMDV 3A.

The FMD viral genome consists of a single open reading frame (ORF) that is translated into a polyprotein and then cleaved into structure proteins (VP0, VP3, and VP1) and nonstructural proteins (L^{pro}, 2A, 2B, 2C, 3A, 3B, 3C^{pro} and 3D polymerase)²². Among these, 3A appears to be a peripheral membrane protein located on the ER with its N-terminus and C-terminus exposed to the cytoplasm²³. The N terminus contains two α -helices responsible for homodimerization and essential for viral replication²⁴, while the C-terminus of 3A is associated with host tropism^{25,26}. However, the detailed mechanism and the biological function of 3A remain largely unknown. Currently, FMDV 3A is only known to be a multifunctional protein that participates in counter-attacking antiviral activity by upregulation of LRRC25 and autophagic degradation of RNA sensors²⁷. In addition to LRRC25, G3BP1, and RNA sensors, the other 3A-associated interaction partners include DDX56, DCTN3 (dynactin subunit 3), vimentin, and RNA helicase A²⁸⁻³¹. Despite intense study, many questions remain, such as the mechanism of LRRC25 upregulation, the biological function of 3A-DCTN3 interaction, and the role of 3A in RO formation.

Protein trafficking from the ER to the ER-to-Golgi intermediate compartment (ERGIC) is mediated by the COPII pathway³². To generate COPII vesicles from the ER, Sar1 is recruited and activated by Sec12 at the ER exit site (ERES). In other words, Sec12 exchanges GDP of Sar1-GDP (inactive form) to GTP, inducing conformational change of Sar1 with the N-terminus attaching to the ER membrane. Active Sar1 recruits the COPII inner coat proteins Sec23 and Sec24, as well as the outer coat proteins Sec13 and Sec31, to bend the membrane into vesicle-like structures³³. Meanwhile, most cargo proteins or cargo receptors can interact with Sec24 to load into the forming vesicles. To form a neck of vesicles, active Sar1 polymerizes. After Sar1 hydrolysis, vesicle scission is complete, and the COPII vesicles are released from the ER toward the ERGIC³³. The opposite direction of transport and the trafficking from the ERGIC to the Golgi is mediated by the COPI pathway instead. Therefore, COPI/COPII machinery is crucial for protein secretion and vital for cells³². It has been found that the machinery is also utilized by enterovirus and FMDV^{8,16}.

In our studies, the topology of 3A was first examined based on homemade monoclonal antibodies (MAbs). Next, we proved that FMDV 3A expression alone could modify ER into a specific vesicle-like structure. With the help of the APEX system, the detailed structure was examined under TEM. Moreover, without the N- or C-terminus of 3A, it would lose ER remodeling, consequently being retained at ER. With preservation of the regions near the hydrophobic region (HR), namely, aa 42–58 and aa 77–92, truncated 3A was sufficient to reshape ER. The modification was associated with two unpublished host partners, Sar1 and Sec12, both of which are COPII factors. The depletion of Sar1 (or Sec12) and the expression of dominant-negative mutants hampered the remodeling. All in all, this is the first study to examine the

features of 3A-induced structures and the mechanism of their formation. We believe these findings could further the investigation of FMDV and other picornaviral RO in the future.

Results

Membrane topology of peripheral FMDV 3A protein

FMDV 3A is a peripheral membrane protein located on the ER, unlike other picornaviruses^{23,34}. However, it was still worthwhile to confirm this with another method. To probe it, we first produced three powerful anti-FMDV 3A MAbs, namely, QA2, PA1, and T10E, against O/TAW/97 (O/99) and O/TAW/97 (O/97) strains. The O/97 strain was a porcineophilic strain with ten amino acid deletion (aa 93–102) in the 3A protein, while 3A of O/99 was relatively conserved to another strain (Fig. S1). Through variant monomeric GFP (mGFP) fused-3A truncation proteins (Fig. 1a), the binding sites for these antibodies were elucidated by western blotting (Fig. 1b). QA2 MAb recognizes the N terminus of 3A (aa 1–41) for O/99 and O/97 virus strains. PA1 and T10E could only interact with the C terminus of 3A for O/99 and O/97 individually (Fig. 1c), a highly diverse region between these two virus strains (Fig. S1). Under digitonin treatment, the antibody could penetrate through the plasma membrane but not the ER or other organelles. For verification, calreticulin, a marker protein located within ER lumen, was detected with Triton X-100 treatment but not with digitonin treatment (Fig. 1d). On the other hand, three anti-3A MAbs could detect 3A in all conditions (Fig. 1e), indicating that 3A was a peripheral membrane protein with both the N- and the C-terminus exposed to the cytoplasm.

FMDV 3A protein modified ER into punctae

Based on colocalization test, O'Donnell *et al.* suggested that FMDV-induced 3A containing vesicles may originate from the ER¹⁵. Gonzalez-Magaldi *et al.* further confirmed a connection between FMDV 3A protein and the ER²³. In our study, we found that the FMDV 3A immunofluorescence signal showed no colocalization with the ER marker protein calreticulin in 3A-transfected PK-15 cells (Fig. S2a), despite the 3A punctae displaying a web-like pattern. To identify the ER structure in live cells without alteration by fixation, we constructed mCherry-ER, in which mCherry was fused with the peptide signal of calreticulin and the KDEL motif (referring to the study by Roderick *et al.*³⁵). A similar strategy has been used in several studies^{36–38}, and the cells were examined by immunofluorescence assay (IFA) (Fig. S2b). After co-expression of mCherry-ER and mGFP-3A, mGFP-3A punctae appeared at the site where the ER collapsed (Fig. 2a). As shown in the right panel of Fig. 2a, the ER structure was disrupted in the area (white arrow) displaced by mGFP-3A punctae, which were linked in a web-like pattern. In the early expression (3 h post-transfection), mGFP-3A showed a web-like structure, but it was only partially colocalized with mCherry-ER (Fig. S2c). Therefore, we strongly suggested that 3A punctae were derived from the ER (Fig. S2d). About 60% mGFP-3A positive cells showed a punctate pattern, while the others appeared as diffuse-type (Fig. S2e). If the C-terminus of 3A were truncated, they all showed a clean reticular pattern and were highly colocalized with mCherry-ER (Fig. 2b). These results support the notion that 3A would locate at the ER while, exerting an unknown function that required the C terminus, 3A

modified ER into punctae. To test the essential region for punctae formation, 3A was truncated into several parts. Considering that GFP would interfere with the structure of truncated 3A, the truncated 3A was fused to the C-terminus of mGFP (or _{2A}eGFP) or the N-terminus of eGFP_{2A} (2A: FMDV 2A peptide, 19 aa) (Fig. 2c & Fig. S2f). As a result, N2HRC1 spanning the region of aa 42–92 was sufficient for punctae formation (Fig. 2c). However, it appeared as a web-like structure with a low expression level (approximately 28% of transfected cells) (Fig. 2d). This result suggested that the ability was dampened but not entirely removed by deleting the N1 and C2 regions.

In addition to 3A, the viral membrane proteins 2B and 2C were also examined. Consistent with previous studies^{17,39}, mGFP-2B would generally be located at the ER, without obvious ER damage (Fig. S2g, left) as compared to mGFP-3A. mGFP-2C also showed colocalization with mCherry-ER and no ER fragmentation at a moderate expression level (Fig. S2g, right).

The 3A punctae were distinct from traditional COPII vesicles or autophagosomes

It has been reported that FMDV induces autophagy in favor of viral replication⁴⁰, and 3A expression upregulates LRR25, an autophagy-related protein²⁷. In addition, FMDV was postulated to initiate viral replication at the sites associated with the ER exit site (ERES), which is responsible for COPII vesicle formation¹⁶. Therefore, we aimed to exclude the possibility that 3A punctae were COPII vesicles or autophagosomes. According to IFA, 3A punctae from 3A(O99) were not colocalized with the COPII inner and outer coat proteins Sec23A and Sec31A (Fig. 3a). Meanwhile, most cells for 3A expression would lead to Sec31A dispersion and downregulation (Fig. 3a & Fig. S3), in concordance with the fact that FMDV infection leads to the dispersal and downregulation of Sec31¹⁶. On the other hand, upon activation of autophagy, LC3B-I would convert into LC3B-II and become evident as punctae (Fig. 3b, c, see starvation condition). Based on the LC3B-II/LC3B-I ratio detected by immunoblotting, 3A protein barely induced autophagy as compared to empty vector transfection (Fig. 3b, c). Because they were not colocalized with mCherry-LC3B (Fig. 3c), it is clear that mGFP-3A punctae were not autophagosomes. These results indicated that 3A punctae were specific structures distinct from COPII vesicles and autophagosomes.

The ultrastructure of the 3A vesicle-like structure in transmission electron microscopy

To gain better insight into the ultrastructure of 3A punctae, we turned to electron microscopy and the APEX system, in which the APEX2 fusion protein could be stained by diaminobenzidine (DAB) directly. First, the patterns of APEX2-3A and APEX2-NHR were similar to mGFP fusion constructs in IFA (Fig. S4a). The APEX2-NHR clearly illustrated the ER structure under electron tomography (Fig. 4a, Video 1). For the full length of 3A, as expected, the ER was disrupted into vesicle-like structures with APEX2-3A around (Fig. 4b; Video 2). The diameter of these structures was heterogeneous and approximately 80.15 ± 26.54 nm (n=83). Similarly, these modified structures were also found in cells expressing APEX2-N2HRC (Fig. S4b) and APEX2-N2HRC1 (Fig. 4c). Moreover, in APEX2-N2HRC1 expressing cells, we also observed dark

reaction products with segmented ER membrane (Fig. 4c, white arrowheads), presumably the state prior to reshaping. All the evidence pointed to 3A curving the ER membrane into vesicle-like structures, and N2HRC1 preserving that ability.

COPII factors, Sar1 and Sec12, were found to be novel 3A interaction partners

Given that polioviral and coxsackieviral 3A interacts with COPI factor GBF1^{6,41} and that FMDV is highly associated with the COPII pathway¹⁶, we tested whether 3A would interact with the COPII factors Sar1 and Sec12. After co-expression of 3A and Sar1 (or Sec12) in a recombinant vaccinia (vTF7-3) transient expression system, the interactions were examined by anti-FLAG co-immunoprecipitation (co-IP). As shown in Figure 5a and 5b, 3A from both O/99 and O/97 virus strains co-immuno-precipitated with recombinant Sar1 and Sec12 from swine and cattle. Therefore, these two novel interactions are likely independent of host species and virus strains. Also, the reciprocal co-IP by PA1 MAb was tested (Fig. 5c). Furthermore, in PK-15 cells co-expressing 3A with sSar1 (Sar1 from swine) or sSec12 (Sec12 from swine), colocalization was detected in double immunofluorescence by anti-FLAG rabbit antibody and QA2 MAb (Fig. 5d).

Next, we tried to map the binding sites on 3A for these two interactions. For Sec12, truncated 3A fragments with N-terminal fusion of mGFP- or C-terminal fusion of -eGFP_{2A} were co-expressed with sSec12 in PK-15 cells. After sSec12 was pulled down, mGFP-HR and C-eGFP_{2A} could be detected, indicating that the binding site for Sec12 on 3A ranged from HR to the C-terminus (Fig. 5e). Further truncation of the C-terminus narrowed this down to the C2 region, aa 93–153 (Fig. 5e, g). For Sar1–3A interaction, truncated GST-3A or 3A-GST were co-expressed with sSar1 in the vTF7-3 expression system, relying on its high production level. The results showed that all three fragments, N-terminus, HR, and C-terminus, were associated with Sar1 (Fig. 5f). More precisely, the region between the N1 and HR regions showed high affinity to Sar1, while the N2 and C1 regions adjoining HR could also interact with Sar1 (Fig. 5f, g).

Knockdown of Sar1 or Sec12 inhibited 3A punctae formation

Because of the high association of Sar1 and Sec12, we reasoned that these two interactions were responsible for 3A punctae formation. To verify that, a knockdown assay was performed. Considering the well-reviewed references for a knockdown in human species and the high abundance of Sar1 protein in PK-15 cells, the human pulmonary cell line A549 was chosen for the knockdown assay. Furthermore, we chose mGFP-N2HRC1 because the weaker reshaping ability of N2HRC1 would be easier to manipulate and observe. It is well known that 3A is a multi-functional protein; the truncation of 3A and removal of its other abilities would help us evaluate the impact on the depletion of Sar1 and Sec12 for punctae formation.

First, Sec12 and Sar1 were knocked down in A549 cells with shRNA or siRNA individually (Fig. S5a). As expected, punctae formation was inhibited in the Sec12 shRNA-A549 cell line (A0 cells) (Fig. 6a). Re-expression of sSec12 restored the vesicular pattern, but not that of Sec12 guanine nucleotide exchange factor-deficient mutant I41A (the mutation was referred to previously ⁴²) (Fig. 6a). These findings indicated that Sar1 activation may be essential for ER remodeling. Similarly, after Sar1 siRNA treatment, the number of punctae decreased in comparison to non-target siRNA treatment, while re-expression of sSar1 could restore punctae formation (Fig. 6b). It is well known that the COPII coat proteins Sec23 and Sec31 catalyze Sar1 GTPase activity for the release of Sar1. Only when the Sar1 activation rate exceeds coat-induced hydrolysis does Sar1 polymerize to form a constricted neck, further exerting vesicle fission dependent on Sar1 hydrolysis. In short, the GTP cycle of Sar1 is essential for COPII vesicle formation⁴³. Therefore, constitutive Sar1-GTP (H79G), constitutive Sar1-GDP (T39N), or defective organization Sar1 (156-QTTG-159 to AAAA) mutants will all block the COPII pathway. In our studies, during the co-expression of msSar1-T39N or msSar1-QTTG (m: mutated) with mGFP-N2HRC1, the formation of 3A punctae is significantly inhibited (Fig. 6c). Nonetheless, msSar1-H79G does not disrupt punctae formation but enhances it (Fig. 6c). In addition, co-IP confirmed that all mutated Sar1 preserved the interaction with 3A (Fig. S5b).

The model of active Sar1-3A intercalating complex for ER remodeling

Based on our mapping analysis, we presumed that FMDV 3A might enhance Sar1 activation by tethering Sar1 to Sec12 with the N-terminus and C-terminus individually. After Sar1 activation, 3A intercalated with two active Sar1 to curve the ER membrane with the N2 and C1 regions (Fig. 7). Therefore, mGFP-N2HRC1 preserved the ability of membrane curvature. In addition, 3A expression resulted in Golgi disassembly (Fig. S6), as also be reported by O'Donnell¹⁵. It is well known that blockage of the COPII pathway results in Golgi disruption due to failure in the transportation of PDIA3⁴⁴. In combination with the 3A induced-Sec31 dispersal (Fig. S3), FMDV 3A should hijack Sar1 and disrupt the function of COPII machinery.

It has been reported that Glycolipid glycosyltransferases (GGTs) directly interacts with Sar1 for transportation from the ER to Golgi⁴⁵. More precisely, the (R/K)X(R/K) motif at the GGTs cytoplasmic tail binds to the pocket around D198, N126, and N94 at Sar1. The D198A mutation of Sar1 abrogates the interaction for GGTs, resulting in the accumulation of GGTs at the ER^{45,46}. However, D198A mutation should not affect most protein trafficking from the ER to the Golgi. Surprisingly, we found that msSar1-D198A led to inhibition of punctae formation (Fig. 8a). Most importantly, D198A mutation significantly decreased the binding ability to the N2 and C1 domains of 3A (Fig. 8b). The D198 residue, the last amino acid of Sar1, was positioned near the membrane, which fits our model.

Discussion

Several advanced studies have revealed that viruses modify host organelles to serve as replication sites or provide viral RNP transport^{1,47-50}. In other words, how viruses modify the host endomembrane is a

current scientific focus, especially in the case of positive-single stranded RNA viruses. Notable studies on enteroviral replication organelles (RO) have been conducted. Although some components, such as ACBD3, PI4K, OSBP, and cholesterol, have proved essential for enteroviral RO, the actual causes of RO formation remain undetermined and debatable^{3,13,51}. Currently, it is believed that viral 3A protein is a key component and one of the initiators. However, FMDV 3A is unique from other picornaviruses²³.

In the study by Gonzalez-Magaldi *et al.*, they utilized a colocalization test, fluorescence recovery after photobleaching (FRAP) analysis, fluorescence loss in photobleaching (FLIP), and membrane bounded fraction analysis to support that 3A was a peripheral ER membrane protein²³. Here, we have demonstrated new evidence showing that FMDV 3A is a peripheral membrane protein, based on digitonin and monoclonal antibodies. Moreover, FMDV 3A modifies the ER into vesicle-like structures, as determined by fluorescent live microscopy and electron tomography with the APEX system. Following up on a study reporting that FMDV was associated with the COPII pathway¹⁶, we found that two COPII factors, Sar1 and Sec12, interact with FMDV 3A based on co-IP and colocalization test, and these two interactions were associated with 3A-induced ER remodeling, as shown by knockdown and re-expression assays. Finally, we built a model to explain how FMDV 3A modifies the ER.

In our hypothesized model, 3A tethers Sar1 to Sec12 for activation using the N-terminus and C2 region. Because of the failure of the active Sar1 pulling down assay, this assumption should be validated in the future. However, Sar1 activation is necessary for remodeling, for functionally deficient Sec12 (I41A-msSec12) would be unable to restore 3A punctae formation in Sec12 depleted cells. After attaching to the ER membrane, two active Sar1 interact with 3A protein in the N2 and C1 regions, causing membrane curvature, instead of recruiting Sec23 and Sec24. Therefore, mGFP-N2HRC1 preserves the ability of ER remodeling. Constitutive active Sar1 (msSar1-H79G) can enhance 3A punctae formation, while constitutive inactive (T39N) and polymerization deficient (156-QTTG-159 to AAAA) Sar1 inhibit that formation. In addition, the 3A expression disrupts the COPII pathway, inducing Sec31 dispersal and Golgi disorganization. Most importantly, D198A mutation of Sar1 inhibits the interaction with the N2 and C1 regions of 3A, thereby inhibiting punctae formation. The D198A mutation does not inhibit Sar1 activation or most protein trafficking that is dependent on COPII vesicles⁴⁶. Because the position of D198 for active Sar1 is located near the membrane, the amino acid fits the positions of the N2 and C1 regions of 3A that adjoin the hydrophobic region.

The results of the knockdown assay also indicated that 3A punctae formation requires Sar1 and Sec12, although we faced numerous difficulties. For example, an attempt to use PK-15 cells in an shRNA strategy was unsuccessful, possibly because of the narrow window between lethal doses of puromycin and the efficient knockdown condition for Sar1 and Sec12. Moreover, we were unable to find available antibodies against endogenous Sec12 from PK-15 cells in western blotting. Therefore, A549 cells were selected for this experiment. In addition, the shRNA Sar1 A549 cell lines could not survive after puromycin selection; therefore, the siRNA strategy was adopted as an alternative for the Sar1 knockdown assay.

Because of biosecurity in Taiwan, our studies were restricted by virus-free regulations, so our hypothesis might be questioned. However, the study by Midgley *et al.* presented exquisite experiments for FMDV infection¹⁶. They showed that knockdown of Sar1 significantly inhibited FMDV infection in IBRS2 cells. The T39N mutation of Sar1 inhibited FMDV infection in IBRS2 cells, while the H79G mutation did not. FMDV infection leads to the dispersal of outer coat protein Sec31 labeling in HeLa cells. All these results showed no conflict with our model's prediction that FMDV 3A utilized and required active Sar1 for ER remodeling, resulting in interference with the COPII pathway. The ER remodeling should be the initiation of RO formation, which is essential for viral replication.

The ER is commonly utilized and modified by viruses such as hepatitis C virus, influenza virus, Zika virus, and reovirus^{36,47,52,53}. Zika virus is a simple but straightforward example of how a virus modifies the ER. Zika NS1 proteins, located within the ER lumen, homodimerize to induce ER invagination, which is likely the same in dengue virus⁵³. However, most other viruses may be more complicated. For example, reovirus-induced ER modification requires both σ NS and μ NS proteins³⁶. For poliovirus (an enterovirus), the modified structure mimetic to RO requires co-expression of 3A and 2BC⁵⁴. We also thought the mechanism of RO formation might be associated with 2B and 2C and required further exploration.

All in all, to our knowledge, this study is the first to show that FMDV 3A protein modifies the ER into vesicle-like structures. The modification is synchronized by two unrecognized interactions, 3A–Sec12 and 3A–Sar1. In combination with other indirect evidence, we presume that 3A enhances Sar1 activation and further interacts with two active Sar1 proteins such that the membrane bends into vesicle-like structures, which may be the first and most important step in RO formation during FMDV infection.

Materials And Methods

Cells and reagents. PK-15 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% FBS. Rapamycin (Sigma, R0395) and Digitonin (Sigma, D141) were purchased. Commercial antibodies used in this study included anti-FLAG mouse antibody (Sigma, F1804), anti-FLAG rabbit antibody (Sigma, F7425), anti- β -actin (Cell Signaling Technology, #3700), anti-GFP (Invitrogen, MA5-15256), anti-calreticulin (LSBio, LS-B9387), anti-LC3B (Cell Signaling Technology, #2775), anti-Sec23A (Novus Biologicals, NBP2-34842), anti-Sec31A (Abcam, ab86600), anti-giantin (Sigma, SAB2100948), anti-Sec12 (LSBio, LS-C29886) and anti-Sar1 (Novus Biologicals, NBP2-20261).

Plasmids. The primers, used in this study, could be found in Supplementary table 1.

(i) 3A associated constructs

To construct expression plasmids pcDNA-3A(O99) and pcDNA-3A(O97), the 3A genes for O/TAW/97 (GenBank No. AY593835.1) and O/TAW/99 (GenBank No. AJ539136.1) were amplified (Phusion™ High-Fidelity DNA Polymerase, Thermo Scientific) from the O/TAW/97 cDNA pool and synthesized correspondingly. The 3A genes were subcloned into pcDNA-3.1(+) using *Hind*III and *Xho*I sites with C-

terminal His tag fusion; the endogenous *NheI* site within O/97 3A was further removed by site-direct mutagenesis without changing the amino acid sequence (GENEART site-directed mutagenesis system, Invitrogen). On the other hand, the mGFP genes were inserted into pcDNA-3.1(+) with *NheI/XhoI* sites, in which a *HindIII* site was inserted between the mGFP gene and *XhoI* site, generating pcDNA-mGFP. The 3A genes from pcDNA-3A(O99) or pcDNA-3A(O97) were subcloned into pcDNA-mGFP by *HindIII/XhoI*, generating pcDNA-mGFP-3A(O99) and pcDNA-mGFP-3A(O97).

The d1D-2A-eGFP (d1A: deleted 1D gene; the last 33 nt of VP1 gene from O/TAW/97) cassette flanked by *NheI-BamHI* and *HindIII-XhoI* was inserted into pcDNA-3.1(+) by *NheI* and *XhoI* restriction enzymes, generating pcDNA-_{2A}eGFP vector. Next, various truncations of 3A genes were inserted between *HindIII/XhoI* (or *HindIII/XbaI*) sites. For 3A-eGFP_{2A} constructs, the genes and restriction sites from pcDNA-_{2A}eGFP were rearranged. The d1D2A gene was subcloned into pcDNA-3.1(+) with *NheI/XhoI* sites, adding *BamHI* site to the 5' end of the d1D2A gene. Next, eGFP gene was inserted into the plasmid by *NheI/BamHI*, and a *HindIII* site was added to the 5' end of the eGFP gene. The variant truncated 3A genes were further inserted by *NheI/HindIII*.

The APEX2 vector was purchased and inserted into pcDNA-3.1(+) by *NheI* and *XhoI*. The indicated truncated or full-length 3A genes were inserted by *XhoI* and *XbaI*. For GST fusion plasmids, GFP genes in variant mGFP- version and -eGFP_{2A} versions were replaced by GST genes using *NheI/HindIII* and *HindIII/XhoI* enzymes respectively.

(ii) Host factor plasmids

The Sar1a and Sec12 cDNA clones from swine and cattle were purchased from Origene (sSar1: NM_001031786; cSar1: XM_005226384; sSec12: XM_003125297; cSec12: NM_001076875). They were inserted into pcDNA-3.1(+) with a FLAG tag at the N-terminus. The sSar1 mutant constructs, including H79G, T39N, and QTTG (156-QTTG-159 to AAAA), were generated using the GENEART site-directed mutagenesis system (Invitrogen) with indicated primers, while D198 was replaced for alanine just by standard PCR with the primers BamHI-sSar1-F and XhoI-msSar1-D198A-R. The mutation of I41A for sSec12 was also performed with a GENEART site-directed mutagenesis system (Invitrogen).

(iii) Others

The mCherry gene, flanked by peptide sequences of calreticulin and KDEL motif, was inserted into pcDNA-3.1(+) by *NheI* and *HindIII* to generate pcDNA-mCherry-ER. The plasmid for expressing mCherry-LC3B was a gift from David Rubinsztein (Addgene plasmid # 40827). The 2B and 2C genes were amplified from the O/TAW/97 cDNA pool and then subcloned into pcDNA-3.1(+) for pcDNA-2B and pcDNA-2C. These two plasmids were further inserted by mGFP genes by *NheI* and *KpnI*, generating pcDNA-mGFP-2B and pcDNA-mGFP-2C.

Monoclonal antibody preparation. BALB/c mice were immunized by purified SUMO-3ABC (O/97) and GST-3ABC (O/99), expressed from *E. coli*. Immunized spleen cells were fused with SP2/0-Ag14 myeloma cells. After 1–2 weeks, hybridoma supernatants were screened by IFA with 3A(O99), 3A(O97), and FMDV

acetone-fixed cell plates. After limited dilution of more than twice, positive clones were further characterized. QA2, PA1, and T10E were selected for this study. Their isotypes all belonged to IgG2a/κ. The purified monoclonal antibodies were obtained as in a previous study⁵⁵. The hybridoma was inoculated intraperitoneally into BALB/c for ascetic fluid and purified by Protein G (GE Healthcare). The purified antibodies were further conjugated with fluorescent dye Dylight488 (Abcam, ab201799) following the manufacturer's instructions.

Western blotting. Equal volumes of cell lysates and elution from co-immunoprecipitation (co-IP) were mixed with sample loading buffer (Bionovas, FA0020) and incubated at 95°C for 5 min. Samples were separated by 13.5% SDS-PAGE, transferred to nitrocellulose membrane (PALL, 79548), and blocked by 5% (w/v) skim milk in PBST at room temperature (RT) for 30 min. The proteins on the membranes were detected by the indicated antibodies overnight in 4°C and corresponding horseradish peroxidase (HRP) conjugated secondary antibodies at RT for 1 h. Given that bands were masked by the light and heavy chains of antibodies used in the co-IP step, secondary antibodies against native antibodies (Abcam, ab131366, which could not recognize mouse IgG1) or specific mouse native antibodies (Abcam, ab131368) were used for elution samples.

Immunofluorescence assay and confocal live-cell imaging. PK-15 or A549 cells, grown on a confocal μ-dish (ibidi, IB-81156), were transfected with indicated plasmids for 24 h. For standard IFA protocols, cells were fixed by 4% paraformaldehyde in PBS for 15 min at 37°C and further permeabilized by 0.5% TX-100 for 5 min at RT. For examination of the 3A protein topology, fixed cells were permeabilized by 50 μg ml⁻¹ digitonin for 5 min at RT. After being washed with PBS three times, samples were incubated with primary antibodies for 2 h at 37°C, followed by incubation with appropriate secondary antibodies for 1 h at 37°C. When double labeling was performed, both antibodies were added together. The nucleus was stained with Hoechst stain (Invitrogen, H3569) for 15 min at 37°C. All imaging was performed under an Olympus IX-83 microscope connected to a CMOS color camera. Live cells were maintained on the microscope stage at 37°C.

Autophagy induction. PK-15 cells in 24-well plates were transfected for 21 h. After washing with PBS, the medium was replaced with fresh DMEM, DMEM with 100 nM rapamycin, or starvation medium (20 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 1% BSA) for 3 h. The cells were collected by 50 μl RIPA buffer (Bio Basic, RB4478); 5 μl of cell lysates were analyzed by 6M urea 13.5% SDS gel, followed by western blotting to detect LC3BI and LC3BII.

Transmission electron microscopy and electron tomography. PK-15 cells were seeded on the Aclar film (Electron Microscopy Science) within 6-well plates. After transfection for indicated plasmids containing APEX2 fusion protein gene for 24 h, the cells were rinsed by PBS, followed by 2% glutaraldehyde fixation in 0.1 M phosphate buffer (PB, pH 7.3) for 1 h on ice. After five times of washing with PBS, 20 mM glycine in PBS was added for 5 min to quench free aldehyde groups. After another five times of washing, cells were incubated with DAB solution with H₂O₂ (5 min for APEX2-3A, 15 min for APEX2-NHR, 30 min for APEX2-N2HRC, and 30 min for APEX2-N2HRC1). The cells were further rinsed in 0.1M PB (2 min x 5

times) and immersed into 2% Osmium tetroxide in 0.1 M PB for 30 min. The cells were then rinsed in distilled water (2 min x 5 times) before overnight staining in a 2% aqueous solution of uranyl acetate. The cells in the monolayer were further subjected to a standard protocol of dehydration and embedding (Spurr's medium) for TEM imaging. The specimen blocks were checked and trimmed under a stereomicroscope, and ultra-thin sections (70 nm) were obtained from the area with positive signals (dark cells). The sections were observed using a transmission electron microscope (TEM, FEI Tecnai G2 TF20 Super TWIN) operating at 120 kV. For electron tomography, serial sections (200 nm) through the APEX2-positive cells were obtained⁵⁶. Double-tilt electron tomography was performed with the TEM (FEI Tecnai G2 TF20) operating at 200 kV. The structures of organelles were depicted by Amira/Avizo software.

Recombinant vaccinia expression system. The protocol followed that of a previous study⁵⁷. HTK⁻ cells were seeded in 6-well plates for 1 day, followed by vTF7-3 infection for production of T7 polymerase within cells for 1 h. The indicated plasmids, containing T7 promoter, were transfected to the cells by using TurboFect (Thermo Scientific). After 20–24 h, cells were ruptured by freeze-thaw cycles for 3 times in 500 µl PBS with protease inhibitor (Millipore, 539134). Finally, the cell debris was removed by 10,000 g centrifugation for 20 min.

Immuno-precipitation assay. For each sample, 1 µg anti-FLAG antibody (Sigma, F1804) or PA1 MAb was incubated with 10 µl Protein G Mag Sepharose (GE Healthcare) in 500 µl binding buffer (50mM Tris, pH 7.5, 150 mM NaCl) with slow end-over-end mixing for 1 h at RT. Cell lysates that came from the vTF7-3 expression system or transfected PK-15 cells were applied to co-immunoprecipitation assay. For the vTF7-3 expression system, a 200 µl sample was incubated with anti-FLAG antibody-magnetic bead overnight after removal of the binding buffer. For transfected PK-15 cells, which were harvested by 200 µl RIPA buffer in each well of 6-well plates, 180 µl samples were applied. After mixing overnight at 4°C, the magnetic bead was washed by PBS or RIPA three times. After replacement with a clean Eppendorf tube, the bead was washed by PBS again. Finally, the sample was eluted by 20 µl 2% SDS and analyzed by western blotting.

Knockdown assay. A549 cells were seeded in a 6-well plate and then transfected with indicated plasmids expressing shRNA, including pLAS2w.Ppuro (empty vector), Sar1-1 (target: CCAGTTCCTAGGACTCTACAA), Sar1-2 (target: CGTGAGATATTTGGGCTTTAT), Sec12-0 (target: GCTGGCCTAAAGATGCAATAA) and Sec12-4 (target: GTGTGCTTCAACCACGATAAT). The plasmids were purchased from National RNAi Core Facility in Taiwan. After 24 h post transfection, cells were selected by 1 µg ml⁻¹ puromycin for 3–4 days and maintained in DMEM with 0.5 µg ml⁻¹ puromycin for more than 2 weeks. For siRNA, cells were seeded in a 24-well plate 1 day before siRNA transfection, in accordance with the manufacturer's instructions for Lipofectamine MessengerMAX Reagent (Thermo Scientific, LMRNA). The target sequence for non-target and Sar1 siRNA was as follows: non-target (UUCUCCGAACGUGUCACGU) and Sar1 (CCAGUUCCUAGGACUCUACAA), purchased from Biotools. To elevate the knockdown efficiency, A549 cells were transfected repeatedly after 24 h siRNA transfection. After 6 h post last siRNA transfection, pcDNA-mGFP-N2HRC1 were transfected to the cells.

Declarations

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Author contributions

I.-V.C. and H.-W.L. conceived the project. Y.-F.J. conducted the experiments for TEM, and the other experiments were designed and performed by H.-W.L.. H.-W.L. and Y.-F.J. wrote the manuscript; H.-W.C. and I.-V.C. revised the manuscript.

Competing interests

The authors declare that there are no conflicts of interest.

Ethical approval

All of the animal experiments were approved and carried out under the regulation and permission of Institutional Animal Care and Use Committee protocols at National Taiwan University (approval number: NTU107-EL-00203), and we abided ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines for reporting animal research.

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Figures

Figure 1

(a) Diagram representing the different truncations of O/99 3A or O/97 3A fused with monomeric GFP (mGFP). We set the alpha-helix2 region and the deletion region in O/97 as the turning points to separate the N- and C-termini into N1/N2 and C1/C2. All the truncation forms here used O/99 normal virus strain as a template, including aa 1–41 deletion (mGFP-N2HRC), aa 1–41 and aa 93–153 deletion (mGFP-N2HRC1), N terminus deletion (mGFP-HRC), C terminus deletion (mGFP-NHR), hydrophobic region (mGFP-HR), C terminus (mGFP-C), and N terminus (mGFP-N). (b) The constructs, expressed in PK-15 cells, were used to examine anti-3A homemade MABs by western blotting. QA2 could not recognize N2HRC, indicating the binding site located at the region within aa 1–41 of 3A, while PA1 recognized the C

terminus of 3A from O/99. The binding site for T10E should be the C terminus of O/97 3A. (2AeGFP: eGFP fused with FMDV 2A peptide in N-terminus) (c) The schematic of the MAbs binding sites on 3A protein. (d) PK15 cells were transfected for expressing 3A from O/99 or O/97. After fixation, the cells were permeabilized by Triton X-100 or digitonin. Anti-calreticulin rabbit antibodies were used to stain the cells in the control group, followed by anti-rabbit secondary antibodies (red) and MAb QA2 conjugated to Dylight 488 (green) for dual labelling. (e) For examination of the topology of 3A, the cells were stained with the indicated MAb (QA2, PA1, or T10E), followed by anti-mouse secondary antibodies. The nucleus was stained with Hoechst stain.

Figure 2

(a, b) Images obtained in PK-15 live cells co-expressed with mCherry-ER and mGFP-3A (or mGFP-NHR). The white arrow indicates the area of ER collapse. (c) Images of variant truncation of 3A, in which the N-terminus was fused to mGFP. The mGFP diffused within the cytoplasm and nucleus. More than half of

the cells expressing mGFP-3A, mGFP-N2HRC, and mGFP-N2HRC1 (60%, 68%, and 72%, respectively) showed multiple punctae in the population. In addition, mGFP-N2HR and mGFP-NHR showed reticular patterns, while only 68% of mGFP-HR expressing cells were reticular, and the others appeared to be diffuse-type. The mGFP-HRC protein distributed diffusely in the cytoplasm, and mGFP-N and mGFP-C, in the cytoplasm and nucleus. (d) N2HRC1 expression in PK-15 cells showing reticular and punctae patterns in low (with longer exposure time) and moderate expression levels. Scale bar, 10 μ m.

Figure 3

(a) PK-15 cells expressing 3A(O99) were stained with specific anti-Sec23A or Sec31A rabbit antibodies, followed by anti-rabbit antibodies conjugated with Alexa Fluor 594. Finally, the 3A(O99) protein was further identified with QA2-Dylight488. (b) PK-15 cells were transfected by empty vector (pcDNA-3.1(+)), pcDNA-mGFP, pcDNA-3A, or pcDNA-mGFP-3A for 21 hr. The cells were further incubated in normal, 100 nM rapamycin, or starvation condition medium for 3 hours. The LC3B proteins from these cell lysates were analyzed by western blotting and quantified in ImageJ software. (c) PK-15 cells co-expressed mCherry-LC3B with mGFP or mGFP-3A. The cells expressing mGFP in starvation condition medium for 3 hours were regarded as a positive control for autophagy activation and showed much more LC3B punctae than did the non-treated cells, but there was no significant increase in mGFP-3A overexpression. Scale bar, 10 μ m. **** $p < 0.0001$.

Figure 4

Electron tomography images of (a) APEX2-NHR and (b) APEX2-3A. The serial section was obtained from PK-15 cells expressing APEX2-NHR or APEX2-3A (full-length of 3A). The ER structures with DAB reaction products for APEX2-NHR (blue) were illustrated in Amira/Avizo software, while eight vesicle-like structures with APEX2-3A were manually illustrated (red) as representative examples. (c) TEM images of APEX2-N2HRC1. White arrows indicate vesicle-like structures with APEX2-N2HRC1. Also, segmented ER was found and marked by DAB reaction (white arrowheads).

Figure 5

The examination for interactions between 3A and Sec12 or Sar1. (a) In the vTF7-3 expression system, HTK cell lysates for co-expression of 3A from O/97 or O/99 and Sec12 from swine or cattle were applied to anti-FLAG co-immunoprecipitation assay. - : empty vector, pcDNA- 3.1(+). none: non-transfection. (b) The examination for Sar1 was performed with the same procedure. (c) Reciprocal co-immunoprecipitation was performed with anti-3A MAb PA1. (d) PK- 15 cells co-expressed 3A(O99) with sSec12 or sSar1. The cells were double-labeled for 3A and recombinant COPII factors by MAb QA2 and anti-FLAG rabbit antibodies, followed by anti-mouse and anti-rabbit secondary antibodies conjugated with Alexa Fluor 647 (green) and Alexa Fluor 594 (red) correspondingly. Scale bar, 10 μ m. (e) PK-15 cells co-expressing sSec12 with variant truncation GFP version for anti-FLAG co-immunoprecipitation. *: residual signal for Sec12. (f) Coexpression sSar1 with the variant truncation GST version in the vTF7-3 system was applied to co- IP. (g) The diagram represents the relative binding affinity of each part for Sar1 or Sec12. The protein level of full-length 3A from the elution was regarded as 100%. Due to a relatively low expression level, HR-GST for Sar1 was excluded.

Figure 6

The fluorescence patterns of mGFP-N2HRC1 in A549 cells were examined in different conditions. (a) mGFP-N2HRC1 was expressed in A549 cells, constitutively expressing control shRNA or Sec12 shRNA. It showed a reticular pattern with punctae for pLAS2w.Ppuro (empty vector) cell lines, while the punctae significantly decreased in the Sec12 shRNA cell line (A0 cells). For re-expression assay, the A0 cells were co-expressed with mGFP-N2HRC1 and sSec12 (wildtype or I41A). The sSec12 proteins were identified by

anti-FLAG mouse antibody ((red). (b) A549 cells were treated with siRNA (non-target; targeting Sar1), followed by mGFP-N2HRC1 expression. For re-expression assay, sSar1 and mGFP-N2HRC1 were co-expressed after Sar1 knockdown, and IFA identified recombinant Sar1 as above. Scale bar, 10 μ m. (c) A549 cells were co-transfected for mGFP-N2HRC1 and dominant-negative msSar1 (mutated sSar1), labeled by anti-FLAG antibodies (red).

Figure 7

In the conventional COPII pathway, after activation by Sec12, Sar1 attaches to the ER membrane with the N-terminus, further recruiting the inner coat proteins Sec23 and Sec24. Sec24 recruits the COPII outer coat proteins Sec31 and Sec13 to bend the membrane into a vesicle-like structure. In FMDV 3A expressing cells, we hypothesize that FMDV 3A modifies the ER into a vesicle-like structure, including Sar1 activation/enhancement and 3A: Sar1-GTP intercalating complex. That is, 3A enhances Sar1 activation. Next, 3A intercalates into two active Sar1 to bend the membrane into a vesicle-like structures.

Figure 8

(a) msSar1-D198A (red) and mGFP-N2HRC1 (green) were co-expressed in A549 cells, followed by IFA for identifying recombinant Sar1 with anti-FLAG antibody. (b) The variant of truncated 3A fused with GST was co-expressed with wild-type sSar1 or the D198A mutation of Sar1. After anti-FLAG co-IP, fragments of 3A were examined by anti-GST antibodies. *: the nonspecific band.

Supplementary Files

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