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Foot-and-mouth disease virus utilizes an autophagic pathway during viral replication

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ABSTRACT

Foot-and-mouth disease virus (FMDV) is the type species of the *Aphthovirus* genus within the *Picornaviridae* family. Infection of cells with positive-strand RNA viruses results in a rearrangement of intracellular membranes into viral replication complexes. The origin of these membranes remains unknown; however induction of the cellular process of autophagy is beneficial for the replication of poliovirus, suggesting that it might be advantageous for other picornaviruses. By using confocal microscopy we showed in FMDV-infected cells co-localization of non-structural viral proteins 2B, 2C and 3A with LC3 (an autophagosome marker) and viral structural protein VP1 with Atg5 (autophagy-related protein), and LC3 with LAMP-1. Importantly, treatment of FMDV-infected cell with autophagy inducer rapamycin, increased viral yield, and inhibition of autophagosomal pathway by 3-methyladenine or small-interfering RNAs, decreased viral replication. Altogether, these studies strongly suggest that autophagy may play an important role during the replication of FMDV.

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Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals caused by foot-and-mouth disease virus (FMDV), the type species of the *Aphthovirus* genus within the *Picornaviridae* family (Grubman and Baxt, 2004). The positive-stranded RNA virus contains a genome of approximately 8400 nucleotides (Grubman and Baxt, 2004).

Infection with positive-strand RNA viruses results in a number of changes in the cell, including the rearrangement of intracellular membranes into vesicular structures where viral genome replication takes place (Armer et al., 2008; Bienz, et al., 1973; Bienz et al., 1987; Egger et al., 2002; Monaghan et al., 2004; Pedersen et al., 1999; Schwartz et al., 2004). Poliovirus, the most extensively studied picornavirus, induces a large number of membranous vesicles, most of them surrounded by double lipid bilayers (Dales et al., 1965; Jackson et al., 2005; Suhy et al., 2000). Characterization of these membranes modified during poliovirus infection demonstrated that they contained cellular markers from the endoplasmic reticulum (ER) as well as membranes from other origins, including lysosomes and the Golgi (Cho et al., 1994; Datta and Dasgupta, 1994; Schlegel et al., 1996; Suhy et al., 2000). Other studies have suggested that several features displayed by the vesicles induced during poliovirus infection, such as double-membrane morphology, vesicular content, and apparent ER origin, are all consistent with an autophagic origin (Schlegel et al., 1996; Suhy et al., 2000; Taylor and Kirkegaard, 2007). Autophagy is a cell-regulated pathway designed to degrade and recycle long-lived proteins and cellular components, an important aspect of organelle turnover and management of starvation (Levine and Klionsky, 2004). Autophagy was first characterized morphologically in mammalian cells; however the molecular components of autophagy were first described in yeast (Wang and Klionsky, 2003). A number of signals can induce autophagy in mammalian cells including starvation, differentiation, development, specific drugs, and infection with intracellular pathogens. During autophagy a double-membrane vesicle, the autophagosome, forms in the cytoplasm sequestering cytoplasmic components or small organelles. The formation and maturation of the autophagosome requires the modification of two ubiquitin-like molecules, microtubule-associated protein light-chain kinase 3 (LC3) and Atg12 (autophagy-related protein) (Espert et al., 2007). Autophagosomes fuse with lysosomes to generate single-membrane vesicles named autophagolysosomes, in which the cargo is degraded. Initial studies provided evidence that the source of the membranes forming this structure was both the Golgi and the ER. However, recent studies suggest that the source of these membranes is primarily from the ER (Dunn, 1990; Espert et al., 2007; Juhasz and Neufeld, 2006). More recently, Hailey et al. (2010) suggested that the mitochondrial outer membrane contributes to their biogenesis during

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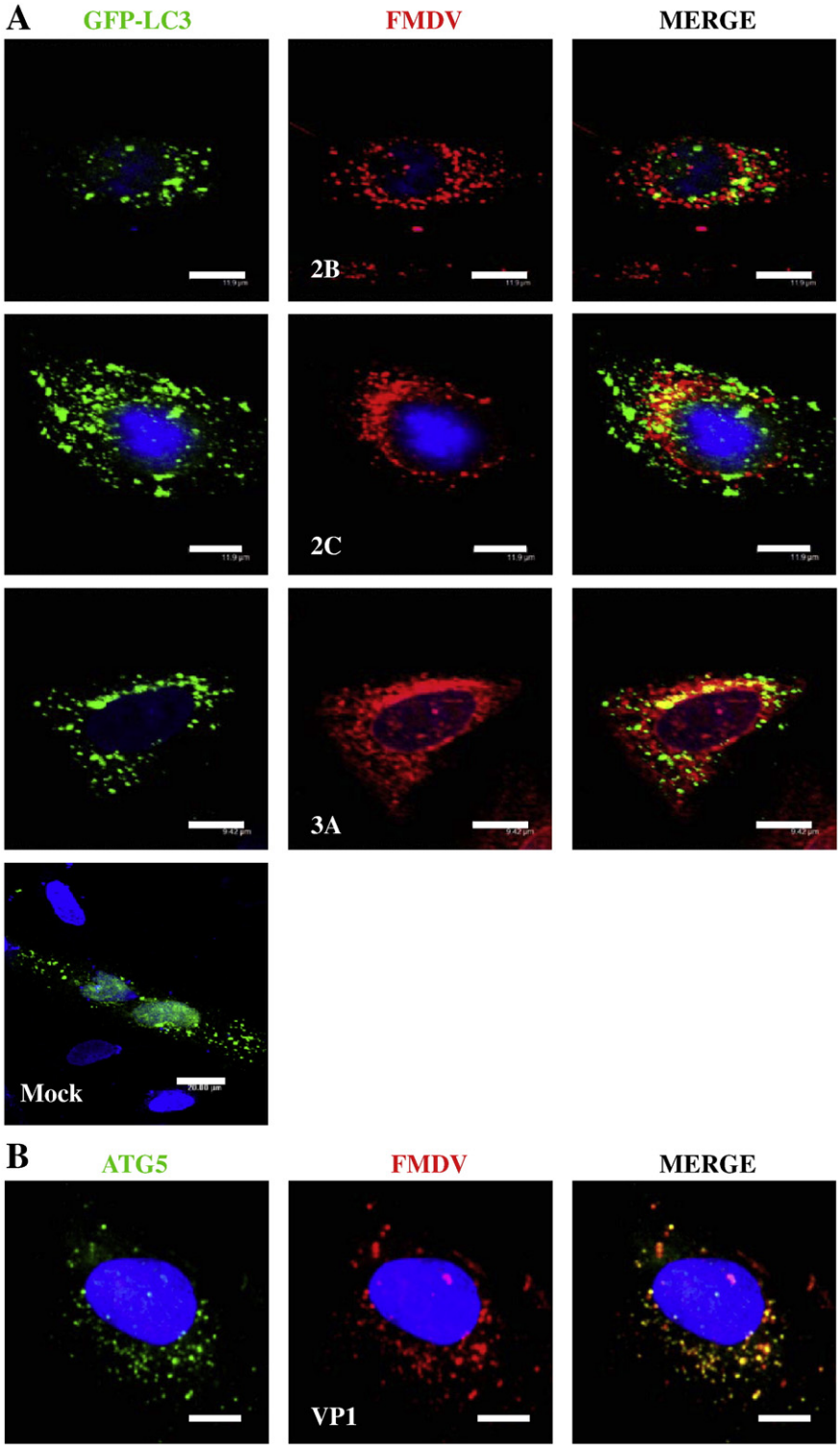

Fig. 1. Analysis of redistribution of autophagy marker GFP–LC3 and FMDV proteins in MCF-10A cells. A. MCF-10A cells were transfected with a plasmid that expresses a GFP–LC3 (green) fusion protein as indicated. Forty-eight hours after transfection, cells were infected with FMDV O_1Campos or mock infected and processed for immunofluorescence staining as described in Materials and methods. FMDV non-structural proteins were detected with specific MAbs and visualized with Alexa Fluor 594 (red). B. MCF-10A cells were infected with FMDV O_1Campos and processed for immunofluorescence staining as describe in Materials and methods. FMDV viral capsid protein VP1, was detected with a MAb and visualized with Alexa Fluor 594 (red); Atg5 was detected with a rabbit antibody and visualized with Alexa Fluor 488 (green).

starvation. The autophagic pathway is also used to degrade intracellular pathogens including bacteria and viruses and may be a component of the innate and adaptive immune responses (Deretic, 2010, Kirkegaard et al., 2004, Lin et al., 2010; Sumpter and Levine, 2010).

Interestingly, recent studies have shown that certain intracellular bacteria and a number of positive-strand RNA viruses, including picornaviruses, induce the formation of autophagic membranes and inhibit either their final maturation or their ability to fuse with lysosomes (Egger et al., 2002; Jackson et al., 2005; Lee et al., 2008;

Pedersen et al., 1999; Schlegel et al., 1996; Suhy et al., 2000). Furthermore the effect of specific autophagy inducers and inhibitors on virus yield supports the idea that autophagosome structures may provide a scaffold for RNA replication by RNA viruses (Dreux et al., 2009; Jackson et al., 2005; Taylor and Kirkegaard, 2007; Wong et al., 2008). These studies also suggested that these double-membrane vesicles, late in infection, may provide a pathway for the non-lytic release of virus (Jackson et al., 2005; Taylor and Kirkegaard, 2007), which would be essential in the establishment of persistent infections, such as that established by FMDV (Alexandersen et al., 2002; Salt, 1993).

In this study, we analyzed the role of autophagy during FMDV replication by using confocal microscopy to monitor the co-localization of specific autophagosome-associated proteins and viral proteins in infected cells. In addition, we explored the effect of inducing or inhibiting the autophagy pathway on FMDV replication. Our results indicate that FMDV non-structural proteins 2B, 2C and 3A, co-localize with the autophagosome marker LC3. Additionally, co-localization of the viral capsid protein VP1 with Atg5 (autophagy-related marker) and LC3 with LAMP-1 in FMDV-infected cells was observed. Furthermore, chemical stimulation or inhibition of the autophagy process directly correlated with increase or decrease in virus production, respectively. Finally, knockdown of LC3 and Atg12, two proteins required to initiate the process of autophagy, led to decreased virus replication. Altogether, these results strongly suggest that autophagy plays an important role in the replication of FMDV.

Results

Redistribution of autophagic markers in FMDV-infected cells

To study if FMDV replication complexes contain constituents of autophagosomes, we used confocal microscopy to monitor the localization of both LC3, a specific marker of autophagosomes, and viral non-structural proteins that have been characterized as critical components of picornavirus replication complex (Bienz et al., 1987; Cho et al., 1994; Jackson et al., 2005; Moffat et al., 2005; Suhy et al., 2000). For most of our experiments we utilized MCF-10A epithelial cells (O'Donnell et al., 2005), unless otherwise indicated. To visualize autophagosomes we expressed LC3 as a fusion product with green fluorescent protein (GFP). In uninfected cells, GFP–LC3 was detected as small discrete foci distributed throughout the cell cytoplasm (Fig. 1A). Upon infection, GFP–LC3 co-localized with FMDV non-structural proteins 2B, 2C, and 3A, by 5 hours post-infection (hpi), adopting a perinuclear pattern (Fig. 1A). By 5 hpi we also show co-localization of the viral capsid protein VP1 with Atg5, an autophagy-related protein that is essential for autophagosome formation (Espert et al., 2007; Matsushita et al., 2007) (Fig. 1B).

To examine the role of autophagy in a system applicable to FMDV pathogenesis studies, we used primary cell cultures derived from bovine pharynx, a site where FMDV primary replication occurs (Arzt et al., 2010; Pacheco et al., 2010a). These cultures, known to be negative for FMDV by virus isolation, RT-PCR and immunostaining (data not shown), were infected with FMDV type O_1Campos at a multiplicity of infection (MOI) of 10. After infection, distribution of viral non-structural protein 3A was compared to the distribution of LC3 by using double-label immunofluorescence and confocal microscopy. Fig. 2 shows a clear co-localization of LC3 and FMDV 3A proteins, with both of the proteins showing a small punctuated distribution pattern.

It has been demonstrated that both LC3 and lysosomal membrane protein 1 (LAMP-1) are components of the mature autophagosome membrane (Espert et al., 2007; Kirkegaard and Jackson, 2005). In order to confirm that autophagy is induced during FMDV replication, we examined the distribution of these two markers in uninfected and infected cells, as well as in the presence of rapamycin, a well-known autophagy inducer. LC3 and LAMP-1 do not appear to co-localize in uninfected untreated cells. Conversely, both in FMDV-infected and in rapamycin-treated cells, LC3 and LAMP-1 proteins clearly co-localize with a perinuclear distribution (Fig. 3), confirming that autophagy occurs in cells infected with FMDV.

Modulation of autophagy activity influences FMDV replication

Well characterized autophagy modulators were used to assess the role of autophagy during FMDV replication in infected cells. FMD viral

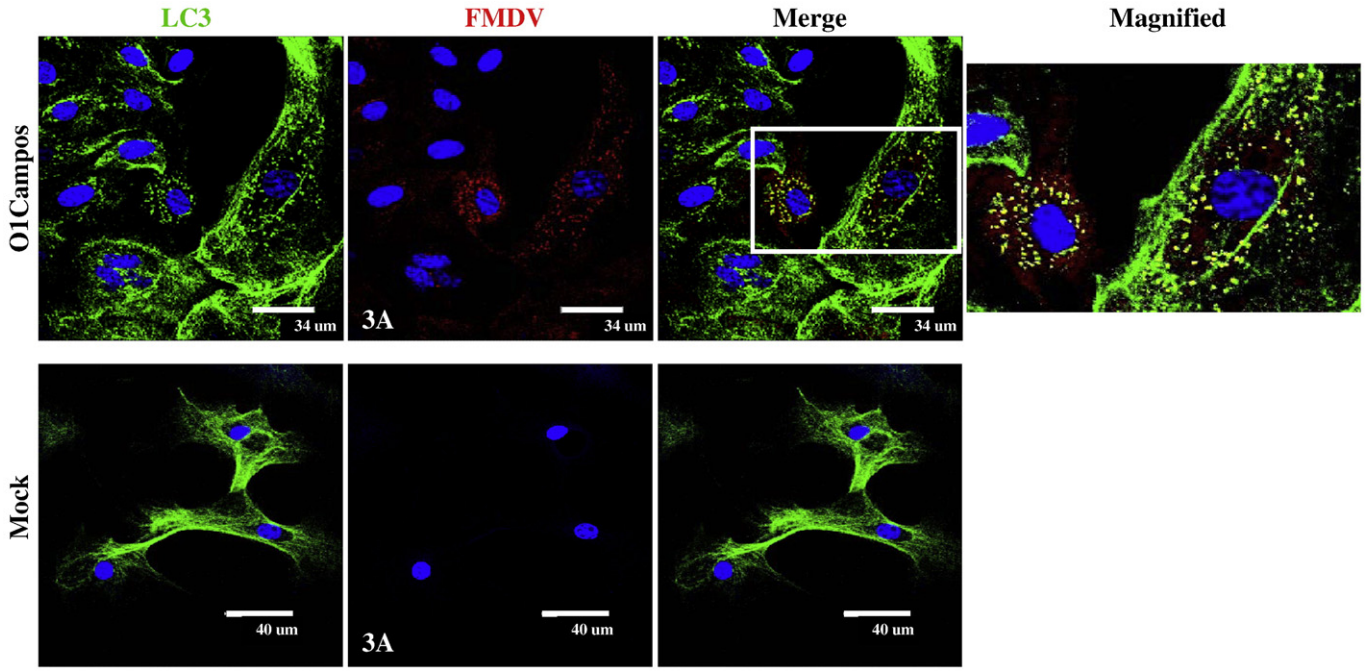

Fig. 2. Analysis of redistribution of autophagy marker LC3 and non-structural FMDV protein 3A in bovine pharynx epithelial primary cultures. Primary cultures of bovine pharynx were infected with FMDV O_1Campos or mock infected and processed for immunofluorescence staining as described in Materials and methods. FMDV non-structural protein 3A was detected with a MAb and visualized with Alexa Fluor 594 (red). LC3 was detected with a rabbit antibody and visualized with Alexa Fluor 488 (green).

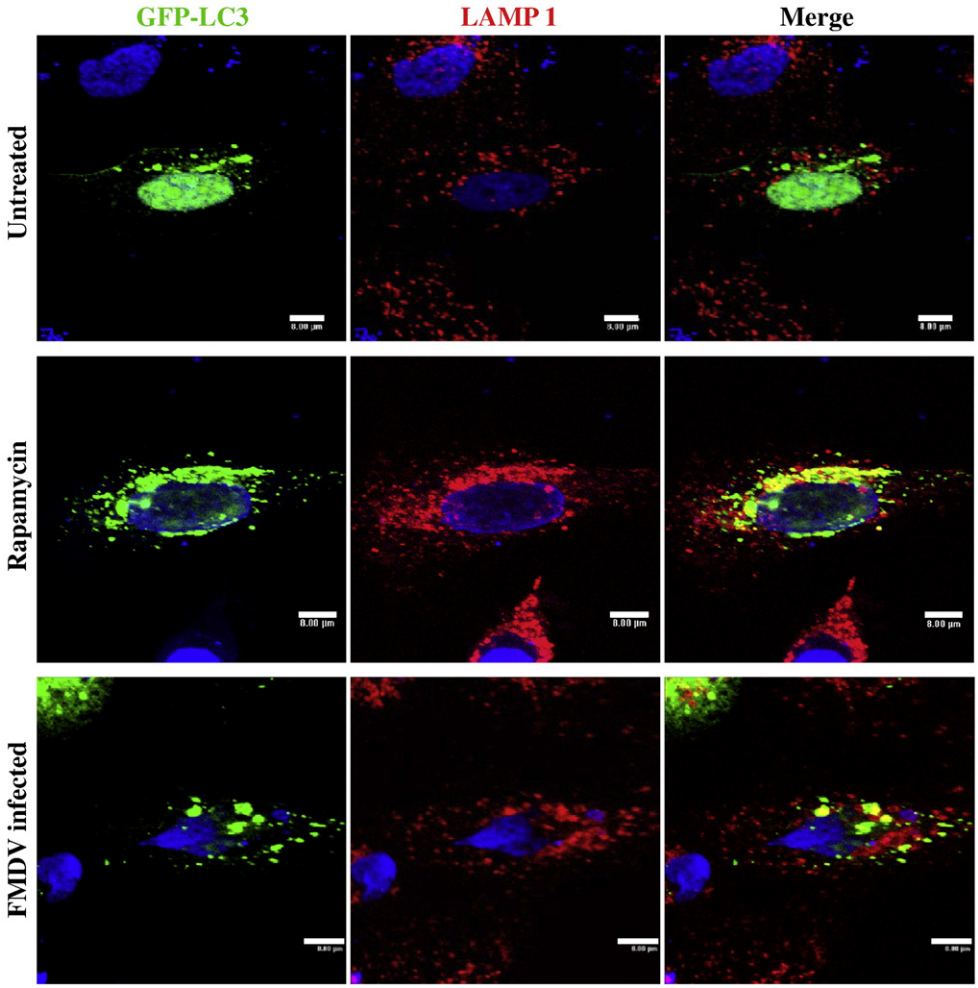

Fig. 3. Detection of GFP–LC3 and LAMP-1 in FMDV-infected cells and in the presence of an autophagy inducer, rapamycin. MCF-10A cells were transfected with a plasmid that expresses a GFP–LC3 (green) fusion protein as indicated. Forty-eight hours after transfection, cells were mock infected or infected with FMDV O_1Campos, or treated with rapamycin. Cells were processed for immunofluorescence staining as described in Materials and methods. LAMP-1 was detected with a MAb and visualized with Alexa Fluor 594 (red).

yield was analyzed in the presence of the autophagy inducer rapamycin or the autophagy inhibitor 3-methyladenine (3-MA). MCF-10A cell cultures were pre-treated either with 50 nM of rapamycin or 20 mM of 3-MA, 2 h prior to infection with FMDV O_1Campos (MOI of 1). Assessment of virus yield was performed at 5 hpi. Yield of virus increased approximately 2 logs when cells were pre-treated with rapamycin (Fig. 4A). Similar results were obtained when cells were infected in the presence of rapamycin and examined by using immunofluorescence and confocal microscopy. At 5 hpi, it was evident that twice the number of rapamycin-treated cells was FMDV-positive when compared to untreated infected cells (data not shown). Conversely, when infected cells were pre-treated with 3-MA,

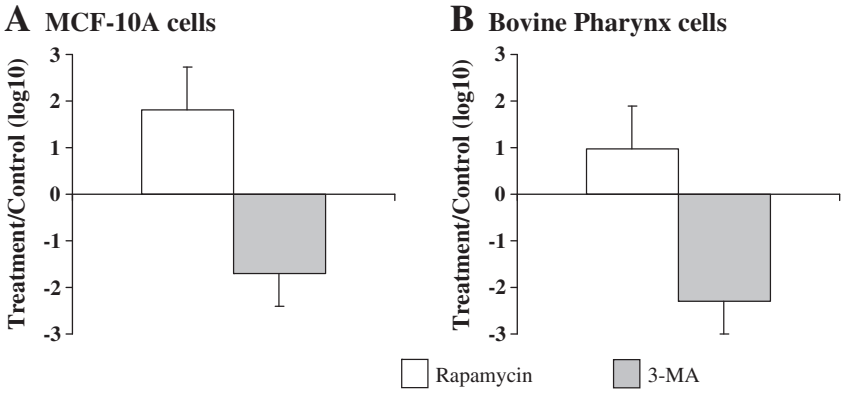

Fig. 4. Viral yields from FMDV-infected cells in the presence of the inducer of autophagy, rapamycin, or the inhibitor of autophagy, 3-MA. MCF-10A cells (A) or bovine pharynx epithelial primary cultures (B) were pre-treated with rapamycin (50 nM) or 3-MA (20 mM) for 2 h before infection with FMDV O_1Campos at an MOI 1, as described in Materials and methods. Viral yields were determined by plaque assay in BHK-21 cells and represented as the ratio of plaque titers of treated and untreated infected cells at 5 hpi. Values are averages and standard deviations representative of two independent experiments.

an autophagy inhibitor, virus yield decreased by nearly 2 logs (Fig. 4A). Moreover, we evaluated the effect of these two compounds in bovine pharynx epithelial primary cultures infected with FMDV O_1Campos. We found that, as observed in MCF-10A cells, when autophagy is induced by rapamycin, viral yield increases up to 1 log. When the FMDV-infected bovine pharynx epithelial cells were treated with 3-MA, viral yield was markedly reduced, more than 2 logs (Fig. 4B). These results suggest that the induction of autophagy during FMDV infection promotes virus replication.

siRNA silencing of the autophagy proteins Atg12 and LC3 results in a reduction of FMDV replication

To confirm the role of autophagy during virus replication, we analyzed the effect of knockdown on transcription levels of autophagy-related proteins LC3 and Atg12. Both proteins have been demonstrated to be critical in the autophagy process (Jounai et al., 2007; Kirkegaard et al., 2004; Kirkegaard and Jackson, 2005; Wang and Klionsky, 2003). When cells were transfected with siRNAs targeting LC3 prior to infection with FMDV O_1Campos, an approximately 3.8-fold and 4.5-fold inhibition in extra- and intracellular viral yield, respectively, was observed (Fig. 5A). Furthermore, an even stronger inhibitory effect on viral replication (greater than 3 logs) was observed when cells were transfected with siRNA targeting Atg12 (Fig. 5B). These results reinforce the hypothesis that autophagy is actively involved in the process of virus replication in cells infected with FMDV.

Increase of cytoplasmic single- and double-membrane vesicles in FMDV-infected MCF-10A cells

Upon induction of autophagy, a membrane cisterna (fold of membrane) known as the isolation membrane (sometimes referred as the phagophore in mammalian cells) appears and curves around part of the cytoplasm. Sealing of the edges of the isolation membrane results in a unique double-membrane vesicle, the autophagosome (Juhasz and Neufeld, 2006). Ultrastructural analysis studies of cells infected with picornaviruses have revealed the presence of virions and other cytoplasmic material within the lumen of double-membrane vesicles (Kirkegaard et al., 2004; Miller and Krijnse-Locker, 2008; Schlegel et al., 1996). To determine whether formation of double-membrane vesicles is linked to autophagy during FMDV infection, electron microscopy was performed on mock and FMDV-infected cells. Quantitative analysis demonstrated an increase in the quantity of the single- and double-membrane vesicles in the FMDV-infected cells (single membrane 160.2 ± 108.2, double membrane 8.1 ± 4.1, and size 0.15 ± 0.04 μm) compared to uninfected cells (single membrane 1.2 ± 3.03, double membrane 0.28 ± 0.89, and size 0.46 ± 0.15 μm) (Fig. 6A). Examination by immunoelectron microscopy (IEM) also showed an increased expression of autophagy-related protein LAMP-1 during virus replication in virus-infected cells compared to uninfected cells (Fig. 6B). Interestingly, IEM also showed a close association of non-structural viral protein 2B with the membranes associated with FMDV infection within the infected cells (Fig. 6B). Therefore, FMDV-infected cells showed evidence of an autophagy-related process, which is absent in mock-infected cells, associated with the expression of non-structural viral proteins.

Discussion

Picornaviruses, as with other positive-strand RNA viruses, generally induce the rearrangement of intracellular membranes to allow the assembly of virus replication complexes (Bienz et al., 1987; Mackenzie, 2005). Co-localization of viral RNA replication complexes with single- or double-membrane vesicles in infected cells has been described several times for various positive-stranded RNA viruses (Bienz et al., 1987; Suhy, et al. 2000; Wong et al, 2008). The double-membrane

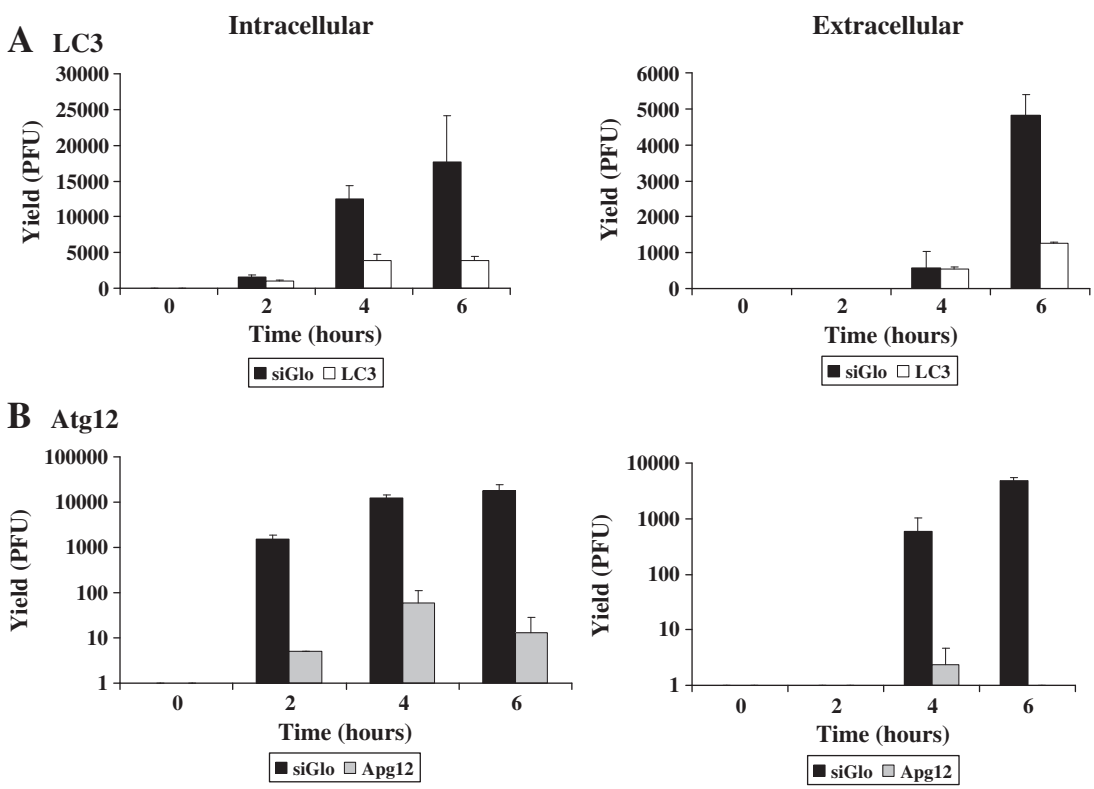

Fig. 5. Viral yields from FMDV infections in MCF-10A cells treated with siRNA to reduce the intracellular concentration of LC3 and Atg12. MCF-10A cells were transfected with four RNA duplexes targeted to LC3 (A), Atg12 (B) or with a control siRNA, siGlo for 48 h at 37 °C as described in Materials and methods. After transfection triplicate plates were infected with FMDV O_1Campos at an MOI of 1 PFU/cell for the indicated times. Plaque titers were determined in BHK-21 cells and expressed as PFU/ml for the intra- and extracellular virus.

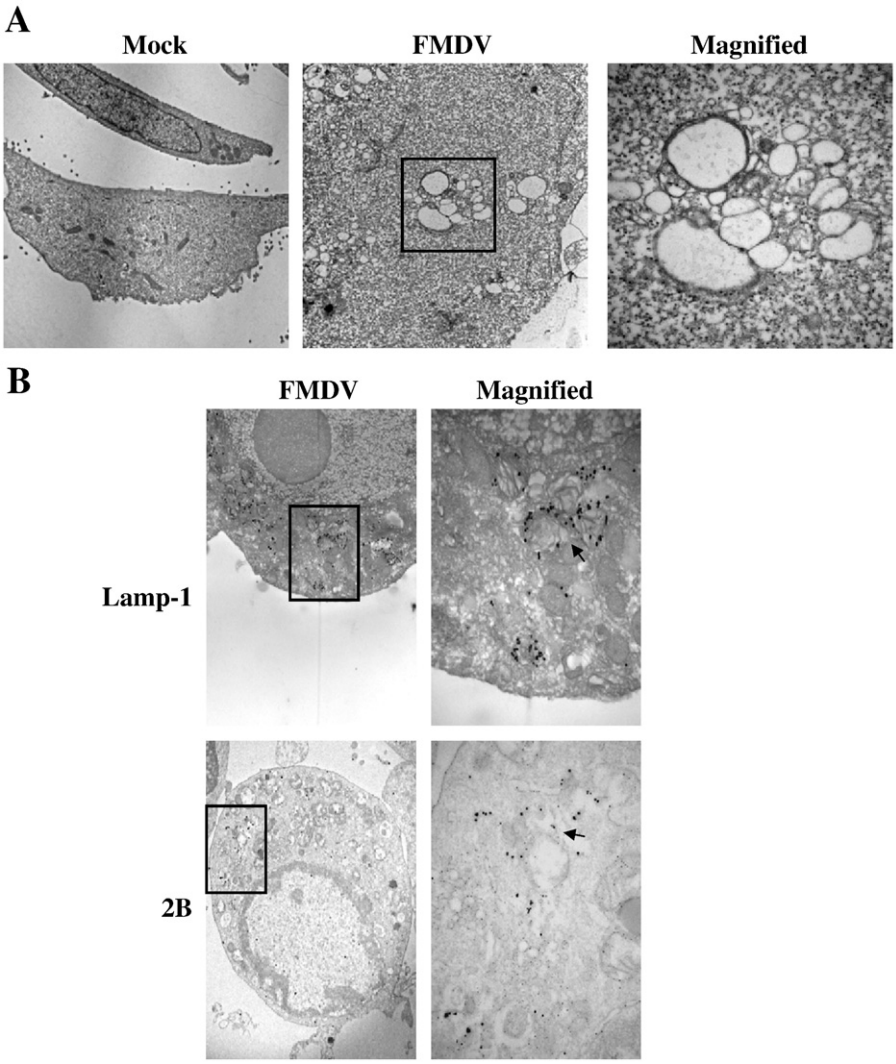

Fig. 6. A — Electron micrographs of mock- and FMDV-infected MCF-10A cells. B — Immunogold detection of LAMP-1 and FMDV 2B non-structural protein individually. LAMP-1 and FMDV 2B proteins were visualized with specific MAb and detected with anti-mouse conjugated with ultra-small gold particles. Note that both proteins are found associated with the infection associated-membranes in the infected cells.

vesicles induced during poliovirus infection shared several features with cellular structures termed autophagosomes, double-membrane vesicles that ultimately mature to degrade their cytoplasmic contents (Dales et al., 1965; Schlegel et al., 1996). Induction of autophagy has been shown to be beneficial for the replication of several viruses including poliovirus, rhinovirus, equine arterivirus, dengue virus, coxsackievirus, hepatitis C virus, and severe acute respiratory syndrome, although the detailed mechanisms remain elusive (Egger et al., 2002; Espert et al., 2007; Jackson et al., 2005; Lee et al., 2008; Pedersen et al., 1999; Wileman, 2006; Wong et al., 2008). Based on these previous findings, it was hypothesized that autophagy may play a role in FMD viral replication. The findings in this report demonstrate that FMDV triggers the autophagy machinery, and this enhances viral replication. This observation is supported by the redistribution of autophagic hallmarks, notably LC3 and LAMP-1, along with virus antigens in FMDV-infected cells, the effect of autophagy modulating compounds on viral yield, and the inhibition of intra- and extracellular virus replication by knockdown of autophagy-related genes.

To understand how autophagy plays a role during FMDV replication, we analyzed the localization of autophagosome markers with viral proteins after infection. We monitored the localization of LC3 with FMD non-structural viral proteins by confocal microscopy, demonstrating that LC3 co-localizes with viral non-structural proteins 2B, 2C and 3A. We also evaluated autophagy in a primary cell culture derived from bovine pharynx, a relevant bovine tissue as a primary site of virus replication (Arzt et al., 2010; Burrows et al., 1981; Pacheco et al., 2010a). Studies performed using bovine pharynx epithelial cell cultures showed a clear co-localization of LC3 and 3A proteins by 5 hpi. For poliovirus the expression of 2BC and 3A in combination, but not separately, induces the formation of double-membrane vesicles that display biochemical markers and fractionate similarly to the vesicles induced during poliovirus infection (Jackson et al., 2005; Suhy et al., 2000). Taylor and Kirkegaard (2007) showed that the expression of the individual poliovirus protein 2BC induces lipid modification of LC3. Conjugation of LC3 to phosphatidylethanolamine (PE) is one of the early steps of autophagosome formation and, it is thought, of vesicle formation during poliovirus infection. In agreement with those results observed for poliovirus infection, within 4 h of FMDV infection, LC3 and LAMP-1, two components of the mature autophagosome membrane, co-localized in both FMDV-infected cells and in rapamycin-treated cells, whereas in uninfected cells no co-localization was seen. Additionally, co-localization of viral capsid protein VP1 with the autophagy-related protein Atg5, was observed (Fig. 1B). Co-localization of autophagosome markers, Atg5 and LC3, with viral replication complexes suggested that at least some RNA replication complexes are present on or near autophagosome-like vesicle.

Co-localization of autophagosome markers and viral proteins could have two explanations. First, the virus could succumb to autophagy as a destructive innate immune response. This has been shown to occur in several viruses and bacteria, including HSV-1, Sindbis Virus, GAS, and TB (Lin et al., 2010; Xu and Eissa, 2010). Some pathogens, however, subvert the cellular autophagy pathway to promote their own replication, including Enterovirus 71 and *Anaplasma phagocytophilum* (Huang et al., 2009; Niu et al., 2008). Wong et al. (2008) have shown that inhibition of signaling pathways or autophagy genes critical for autophagosome formation reduces coxsackievirus B3 (CVB3) viral protein expression and the viral progeny titer. Conversely, increasing the cellular number of autophagosomes prior to viral infection by cellular starvation, rapamycin treatment, or lysosomal inhibition promotes viral replication, suggesting that the host double-membrane autophagosome is likely utilized by CVB3 to facilitate its own replication (Wong et al., 2008).

Poliovirus also subverts the autophagy pathway, inducing structures resembling cellular autophagosomes on which viral RNA replication complexes are assembled. Treatment with either rapamycin or tamoxifen, both inducers of autophagy, increased poliovirus yield 3–5-fold (Jackson et al., 2005). Therefore, autophagosome formation increases poliovirus load suggesting that the autophagic machinery serves a valuable role in viral replication (Jackson et al., 2005).

Accordingly, in our study, viral loads increased when MCF-10A cells as well as the bovine pharynx epithelial primary cells were infected with FMDV in the presence of the autophagy inducer rapamycin (Figs. 4A–B). The increase of viral yield was manifest to a greater extent in the extracellular viral yield as opposed to intracellular virus in MCF-10A cells (data not shown). Our results clearly indicate that FMDV-induced autophagic machinery is favorable for FMDV replication. In contrast, when autophagy is blocked in both cell types by 3-MA, viral loads are reduced (Figs. 4A–B). Interestingly, when autophagy is inhibited by 3-MA, FMDV replication still continues at a reduced level, suggesting that the autophagy machinery may play a promoting role in the replication of FMDV rather than being a critical factor. Based on these results, it is therefore likely that the autophagic machinery does not have a destructive role during FMDV infection and instead contributes to the formation of viral replication complexes.

To further examine the role of autophagy, we tested the effect of LC3 and Atg12 knockdown on FMDV replication. By using siRNA to reduce the amount of intracellular autophagy proteins LC3 and Atg12, an inhibitory effect on viral replication was detected, which was more significant in extracellular than in intracellular viral yields, indicating that the expression of autophagy-related proteins is important and necessary for the virus. Jackson et al. (2005) also reported a larger effect on extracellular than intracellular virus yield when the abundance of autophagy proteins Atg12 and LC3 was reduced by siRNA. These authors suggest that the reduction in autophagosome machinery decreased cell lysis early in infection, or that reduced abundance of autophagosomal machinery decreased non-lytic viral escape, contributing to the decrease in extracellular virions. We show here a similar effect on FMDV, indicating that inhibition of autophagy has a stronger effect on extracellular than intracellular viral yields.

EM analyses of cells infected with poliovirus showed the presence of large numbers of membranous vesicles with diameters of 200–400 nm, which due to the 'cytoplasmic matrix' present in the lumen of the vesicles, were postulated to develop by a mechanism comparable to that of the formation of "autolytic vesicles" (Dales et al., 1965; Schlegel et al., 1996). As mentioned above, positive-strand RNA viruses, such as poliovirus, equine arterivirus and murine hepatitis virus, all require membrane surfaces on which to assemble their RNA replication complexes (Mackenzie, 2005). For instance, in poliovirus-infected cells the membranes that are induced during infection resemble autophagosomes due to the double-membrane-bound morphology that is present even early during the infection, their specific labeling with anti-LAMP-1 antibodies, and their low buoyant densities (Jackson et al., 2005; Suhy et al., 2000). These facts obtained using other RNA viruses support our observations regarding membranous vesicles that resemble autophagosomes and their role during FMDV replication. MCF-10A cells infected with FMDV demonstrated an increase of both single- and double-membrane vesicles, as shown in Fig. 6, which are associated with the autophagy-related protein LAMP-1 as well as FMDV non-structural protein 2B. It has been reported that 2BC expression leads to the accumulation of large single-membrane vesicles (Cho et al., 1994; Suhy et al., 2000) with buoyant densities similar to those of the vesicles induced during poliovirus infection (Suhy et al., 2000). Further investigation of the ultrastructure and biochemistry of these vesicles formed during autophagy and the expression of individual viral proteins may reveal additional steps to the lipid sequestration, cytosolic wrapping, and maturation of these unique intracellular compartments. A hypothesis for the presence of autophagosome-like membranes in poliovirus-infected cells is that these membranes are induced as a component of the innate immune response (Jackson et al., 2005). Autophagy is increasingly appreciated as a functional pathway in the innate immune response to several viruses, intracellular bacteria, and parasites. The specific mechanism by which the autophagosome is utilized during replication in host cells has not yet been elucidated, however it is likely that the autophagosome provides a physical scaffold where the virus complex may reside as has been suggested for poliovirus and coxsackievirus (Jackson et al., 2005; Taylor and Kirkegaard, 2007; Wong et al., 2008).

This report constitutes the first demonstration that autophagy appears to be an important part of the FMDV replication cycle, although the mechanism by which FMDV infection induces the activation of autophagic machinery remains to be determined. The correlation between the autophagy pathway and increase of viral yield supports this observation. It could be speculated that autophagy may be a possible mechanism FMDV uses to maintain a persistent infection. A number of studies have shown the importance of the pharyngeal area tissues during persistent FMDV infection (Burrows et al., 1971, 1981; Prato Murphy et al., 1994; Zhang and Kitching, 2001). The evidence of autophagic machinery activation in FMDV-infected bovine pharynx epithelial primary cultures, a relevant tissue during persistent infection in the natural host, may indicate that under certain circumstances, autophagy enables the non-lytic release of the virion from persistently infected cells.

Materials and methods

Cell lines, virus and plasmid

Human mammary gland epithelial cells (MCF-10A) were obtained from ATCC (catalogue no. CRL-10317) and maintained in a mixture of Dulbecco's minimal essential medium (DMEM, Invitrogen, CA) and F12 Ham media (1:1, Invitrogen) containing 5% heat-inactivated fetal bovine serum (HI-FBS, HyClone), 20 ng/ml epidermal growth factor (Sigma, St.Lois, MO), 100 ng/ml cholera toxin (Sigma), 10 μg/ml insulin (Sigma), and 500 ng/ml hydrocortisone (Sigma).

FMDV type O_1 strain Campos (O_1Campos) was derived from the vesicular fluid of an experimentally infected steer. The virus was grown in baby hamster kidney-21 (BHK-21) cells and the titer determined by plaque assay on (BHK) cells using standard techniques (Pacheco et al., 2010b). Plasmid designed to express GFP–LC3 fusion protein was produced and described elsewhere (Jackson et al., 2005). Bovine pharynx epithelial primary cultures were produced by using a modification of preparing keratinocyte cultures and described elsewhere (Barlow and Pye, 1989; O'Donnell et al., 2001).

Antibodies and reagents

Monoclonal antibody (MAb) 12FB, directed against the FMDV type O_1 structural protein VP1, has been previously described (Stave et al.,

1986). Monoclonal antibodies to the non-structural FMDV proteins 2B, 2C and 3A, were obtained from Dr. E. Brocchi, Istituto Zooprofilattico Sperimentale della Lombardia e dell Emilia-Romagna, Brescia, Italy. Rabbit antibodies to LC3 (AnaSpec) and Atg5 (Santa Cruz) were used as autophagy markers. Anti-LAMP-1 monoclonal antibody (BD Biosciences) was used to detect LAMP-1, a marker of late endosomes and lysosomes. An autophagy inducer, rapamycin (Cell Signaling Technology, Beverly, MA), prepared as a 100 μM stock solution in methanol, was used at a 50 nM concentration diluted in MEM media, and the inhibitor 3-methyladenine (3-MA, Sigma, St Louis), was used at a 20 mM concentration diluted in MEM medium.

Infection and transfection of cells and confocal microscopy

Sub-confluent monolayers of MCF-10A cells or bovine pharynx epithelial primary cultures grown on 12 mm glass coverslips in 24-well tissue culture dishes were infected with FMDV O_1Campos at an MOI of 10 PFU/cell, for 1 h at 4 °C in MEM containing 0.5% HI-FBS and 25 mM HEPES, pH 7.4. After the 1 h adsorption period the supernatant was removed and the cells rinsed with ice-cold 2-morpholinoethanesulfonic acid (MES) buffered saline (25 mM MES, pH 5.5, 145 mM NaCl) to remove unadsorbed virus. The cells were washed once with media before fresh media was added, then incubated at 37 °C. To express GFP–LC3 protein, monolayers of MCF-10A cells were transfected with 1 μg of plasmid DNA by using FuGene (Roche, Mannheim, Germany), following the manufacturer's recommendations. At 24 h post-transfection the cells were infected as described above, fixed with 4% paraformaldehyde (EMS, Hatfield, PA) at the appropriate times and analyzed by confocal microscopy. To study whether rapamycin (50 nM) or 3-MA (20 mM) affected viral replication, cells were pre-incubated with each compound for 2 h at 37 °C, then subsequently infected with FMDV O_1Campos (MOI 1). Immunofluorescence and confocal microscopy using the antibodies listed above were performed as previously described (O'Donnell et al., 2005). In order to get a representative image for each experiment, we analyzed 5 to 10 independent fields examining 15–20 cells/field at a low magnification (40×) and an additional observation at higher magnification (63×) where variable numbers of fields with 2–3 cells/field were examined. Of note, each of the experiments shown in Figs. 1, 2, 3 and 6 was run at least 3–4 independent times.

Viral replication in the presence of compounds

MCF-10A cells or bovine pharynx epithelial primary cultures were incubated with the compounds at the concentrations listed above for 2 h at 37 °C prior to infection. The cells were then infected with FMDV O_1Campos at an MOI of 1 in the presence of either rapamycin or 3-MA. At the end of the adsorption period the supernatant was removed, followed by a single rinse with ice-cold MES buffered saline to inactivate unadsorbed virus. The cells were washed once with media, before fresh media with or without the rapamycin or 3-MA was added. One set of cultures was immediately frozen at −70 °C, and the other set of plates was incubated for an additional 4 h at 37 °C, and then removed to −70 °C. The plates were then thawed and cell debris was removed by centrifugation and viral titer was determined by plaque assay on BHK-21 cell monolayers.

Knockdown of autophagy genes by RNA interference

siRNA SMARTpools consisting of four RNA duplexes targeting the gene of interest (Jackson et al., 2005) and a control siRNA (siRNA Glo) were purchased from Dharmacon (Lafayette, CO). For LC3, both LC3A and LC3B were targeted, bringing the total number of transfected duplexes to eight. MCF-10A cells were grown to densities of 1.0×10^5 cells per well in 24-well tissue culture dishes in 1 ml of media without antibiotics, and transfected using Lipofectamine 2000 (Invitrogen) as previously described (Jackson et al., 2005). Forty-eight hours after transfection, the cells were infected with FMDV O_1Campos at an MOI of 1 for 1 h at 37 °C. After adsorption, the inoculum was removed, and the cells were rinsed with ice-cold MES to remove residual virus particles, rinsed with MEM containing 1% FBS and 25 mM HEPES, pH 7.4, then incubated at 37 °C. At appropriate times post-infection, the cells were frozen at −70 °C, and the thawed lysates were used to determine titers by plaque assay on BHK-21 cell monolayers.

Electron and immunoelectron microscopy

Sub-confluent monolayers of MCF-10A cells grown on tissue culture dishes (MatTek, Ashland, MA) were infected with FMDV O_1Campos, at an MOI of 10 PFU/cell for 1 h at 4 °C in MEM containing 0.5% FBS and 25 mM HEPES, pH 7.4. At the end of the adsorption period the supernatant was removed and the cells were rinsed once with ice-cold MES to remove unadsorbed virus. The cells were then washed once with media followed by addition of fresh media, and incubated at 37 °C for 4 h. At this time, the media was removed and the cells were fixed with a solution containing 2.5% glutaraldehyde, 100 mM NaCl, and 2 mM calcium chloride, in HEPES buffer, pH 7.4 for 1 h at room temperature (RT). The cells were rinsed twice with a solution containing 100 mM NaCl, 2 mM calcium chloride, and 3.5% sucrose in HEPES buffer, pH 7.4 and post-fixed with 1% osmium tetroxide and 1.5% potassium ferricyanide. The cells were stained *en bloc* with 2% aqueous uranyl acetate overnight, dehydrated with ethanol and infiltrated with EmBed 812 resin (EMS). To view sequential organization of the replication complexes and other membrane vesicles, ribbons of 70 Angstrom sections were picked up on slot grids and grid stained with methanolic uranyl acetate and lead citrate. The sections were examined with a Hitachi T-7600 electron microscope operated at 80 kV. Digital images were captured with an Advanced Microscopy Techniques camera (Danvers, MA). For *in situ* IEM analysis, FMDV-infected and mock-infected MCF-10 cell monolayers were fixed in the MatTek dishes using a protocol described elsewhere and ultra-small gold and other reagents from Aurion (Wageningen, Netherlands) were used. Briefly, the cell monolayers were fixed using a solution containing 1% lysine, 4% paraformaldehyde (electron microscopy grade 16%) (EMS), 1% sodium meta-periodate and 0.1% glutaraldehyde in HEPES buffer, pH 7.4 for 1 h at RT. Primary antibodies were incubated overnight at 4 °C. The cell monolayers were rinsed extensively in binding buffer and incubated with an anti-mouse conjugated to ultra-small gold (dil 1/300, Aurion, EMS) for 1 h at RT. The cell monolayers were rinsed 5 times with binding buffer, 2 times with Dulbecco's Phosphate buffered saline (PBS) and fixed with 2% glutaraldehyde in PBS. After extensive washing with MilliQ water (Millipore), the ultra-small gold was enlarged to approximately 10 nm using Gold En-hance (Aurion) for 20 min at RT. The cell monolayers were incubated with a solution of 0.5% osmium tetroxide for 30 min at 4 °C on wet ice, rinsed with water, dehydrated with ethanol and embedded in Spurr's Resin per manufacturer's instructions.

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