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Cholesterol shuttling is important for RNA replication of coxsackievirus B3 and encephalomyocarditis virus

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Summary

Picornaviruses are a family of positive-strand RNA viruses that includes important human and animal pathogens. Upon infection, picornaviruses induce an extensive remodelling of host cell membranes into replication organelles (ROs), which is critical for replication. Membrane lipids and lipid remodelling processes are at the base of RO formation, yet their involvement remains largely obscure. Recently, phosphatidylinositol-4-phosphate was the first lipid discovered to be important for the replication of a number of picornaviruses. Here, we investigate the role of the lipid cholesterol in picornavirus replication. We show that two picornaviruses from distinct genera that rely on different host factors for replication, namely the enterovirus coxsackievirus B3 (CVB3) and the cardiovirus encephalomyocarditis virus (EMCV), both recruited cholesterol to their ROs. Although CVB3 and EMCV both required cholesterol for efficient genome replication, the viruses appeared to rely on different cellular cholesterol pools. Treatments that altered the distribution of endosomal cholesterol inhibited replication of both CVB3 and EMCV, showing the importance of endosomal cholesterol shuttling for the replication of these viruses. Summarizing, we here demonstrate the importance of cholesterol homeostasis for efficient replication of CVB3 and EMCV.

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Introduction

The *Picornaviridae* constitute a large family of positive-strand RNA [(+)RNA] viruses that includes many important human and animal pathogens. Picornaviruses have been implicated in a wide range of acute and chronic conditions such as poliomyelitis (poliovirus), meningoencephalitis and myocarditis (coxsackie and echoviruses), respiratory tract illnesses ranging from common cold to asthma exacerbation and chronic obstructive pulmonary disease (rhinoviruses), foot-and-mouth disease (foot-and-mouth disease virus) and hepatitis A (hepatitis A virus).

All (+)RNA viruses replicate their genome in the cytoplasm of the host cell, which is associated with extensive remodelling of intracellular membranes into membranous replication organelles (ROs) (Belov and van Kuppeveld, 2012). The exact role of this membrane reorganization is unknown, but it has been suggested to support the replication process in a number of ways: by providing a scaffold for the replication machinery, by increasing the local concentration of viral and cellular factors required for replication and by confining the RNA replication process in a specific location protected from the innate immune system (Miller and Krijnse-Locker, 2008; Nagy and Pogany, 2012). Picornaviruses from different genera require disparate sets of host proteins to support their replication. Enteroviruses, such as coxsackievirus B3 (CVB3) and poliovirus, were shown to require GBF1 and PI4KIIIβ for efficient replication and to be sensitive to GBF1 and PI4KIIIβ inhibitors (Maynell *et al.*, 1992; Belov *et al.*, 2007; Lanke *et al.*, 2009; Hsu *et al.*, 2010), whereas cardioviruses [e.g. encephalomyocarditis virus (EMCV)] were reported not to be inhibited by GBF1 and PI4KIIIβ inhibitors (Gazina *et al.*, 2002; van der Schaar *et al.*, 2013). Furthermore, CVB3 has been proposed to replicate on Golgi-derived membranes, whereas EMCV is thought to replicate on endoplasmic reticulum (ER)-derived membranes (Gazina *et al.*, 2002). Until now most studies have focused on the role of viral and host proteins in the membrane remodelling process, but the role of lipids remains poorly characterized. Recently, phosphatidylinositol-4-phosphate (PI4P) was the first lipid discovered to be important for the replication of a

number of enteroviruses (Hsu *et al*., 2010). PI4P was suggested to be important for the recruitment and stabilization on the membranes of the RNA-dependent RNA polymerase 3Dpol. Furthermore, the PI4P lipids may recruit other host factors involved in genome replication.

Studies on other (+)RNA viruses, e.g. hepatitis C virus, have revealed a role not only for PI4P in virus replication and membrane remodelling (Hsu *et al*., 2010; Reiss *et al*., 2011) but also for the membrane lipid cholesterol (Sagan *et al*., 2006; Honda and Matsuzaki, 2011). Free, i.e. unesterified, cholesterol is an essential lipid for various cellular structures and processes as it is important for the fluidity and impermeability of cellular membranes and, due to its importance for lipid rafts formation, plays a key role in signal transduction. Therefore, cholesterol homeostasis is under tight control. Cholesterol does not statically reside in cellular membranes, but is actively shuttled between cellular compartments. The endosomal system plays an important role in this cholesterol shuttling as it receives endocytosed cholesterol from the plasma membrane and distributes it to different organelles, including plasma membrane, Golgi apparatus and ER (Ikonen, 2008). Cholesterol was shown to be important for entry and replication of dengue virus and for the cellular antiviral response in West Nile virus infections (Mackenzie *et al*., 2007; Rothwell *et al*., 2009; Poh *et al*., 2012). In the case of picornaviruses, cholesterol has mainly been linked to virus entry (Danthi and Chow, 2004; Coyne and Bergelson, 2006; Wong *et al*., 2006). Recently, Ilnytska *et al*. (2013) showed that enteroviruses require endocytosed cholesterol for efficient replication, enhance cholesterol uptake to provide their needs and recruit recycling endosomes to deliver endocytosed cholesterol to their ROs.

In this study, we focus on the role of cholesterol in the replication of CVB3 and EMCV. We found that EMCV, although dependent on different host factors than CVB3, also recruits and requires cholesterol for replication. We also investigated whether CVB3 and EMCV depend on a certain cholesterol pool using two related β-cyclodextrins (βCDs) that were implied to differentially deplete cholesterol from intracellular compartments (Zidovetzki and Levitan, 2007). Remarkably, we found differential effects on virus replication when reducing cholesterol to similar levels, suggesting that the two viruses rely on distinct cholesterol pools. Furthermore, we show that not the total cholesterol content but rather endosomal cholesterol homeostasis is important for replication. Finally, we show that a CVB3 mutant that can replicate independent of PI4KIIIβ, a protein that was suggested to play a role in the recruitment of cholesterol-rich endosomes to ROs, still requires cholesterol for replication.

Results

Free cholesterol is redistributed to CVB3 and EMCV ROs

We studied the distribution of cholesterol in cells infected with CVB3 or EMCV, two picornaviruses from different genera. We visualized cholesterol using filipin, a fluorescent polyene macrolide that specifically binds free cholesterol, and we used antibodies against the CVB3 3A protein and the EMCV 3AB protein to mark ROs. We first ruled out cross-talk between filipin and the fluorescent dye conjugated to the secondary antibodies by performing single stainings (data not shown). In uninfected cells, filipin stained the plasma membrane and perinuclear region (Fig. 1A and B). Upon infection with CVB3, the normal filipin staining pattern was lost, indicating cholesterol was redistributed to confined structures in the cytoplasm where it colocalized with the viral 3A protein [Fig. 1A, Manders' colocalization coefficient (MCC) 0.64]. In EMCV-infected cells, cholesterol was redistributed to confined structures in the cytoplasm where it colocalized with 3AB (Fig. 1B, MCC 0.78). Thus, in both CVB3 and EMCV-infected cells cholesterol is redistributed to the ROs.

Cholesterol depletion differentially affects CVB3 and EMCV replication

Next, we wanted to know whether cholesterol is required for genome replication of CVB3 and EMCV. Cholesterol is present at highest concentrations in the plasma membrane but is also found in a number of other cellular compartments, forming different pools that are in continuous exchange with each other. When added to the culture medium, βCDs can only extract cholesterol from the plasma membrane, but due to the continuous exchange between cholesterol pools they also deplete intracellular cholesterol. Although it is thought that all βCDs complex cholesterol in a similar manner, the related cyclodextrins methyl-β-cyclodextrin (MβCD) and hydroxypropyl-β-CD (HPCD) were suggested to differentially deplete cholesterol from intracellular compartments (Zidovetzki and Levitan, 2007). We investigated whether CVB3 and EMCV have a preference for different cholesterol pools by depleting with MβCD and HPCD. To prevent saturation of cyclodextrin with cholesterol from the culture medium, we replaced foetal calf serum (FCS) with lipoprotein-deficient serum (LPDS). We assayed virus replication using recombinant viruses that express *Renilla* luciferase (RLuc-CVB3 or RLuc-EMCV) and quantified the amount of luciferase produced as a measure of replication (Fig. 2A and B). In parallel, we measured total cholesterol levels to check the efficiency of cholesterol depletion. Although 0.25% MβCD and 1% HPCD reduced cholesterol to

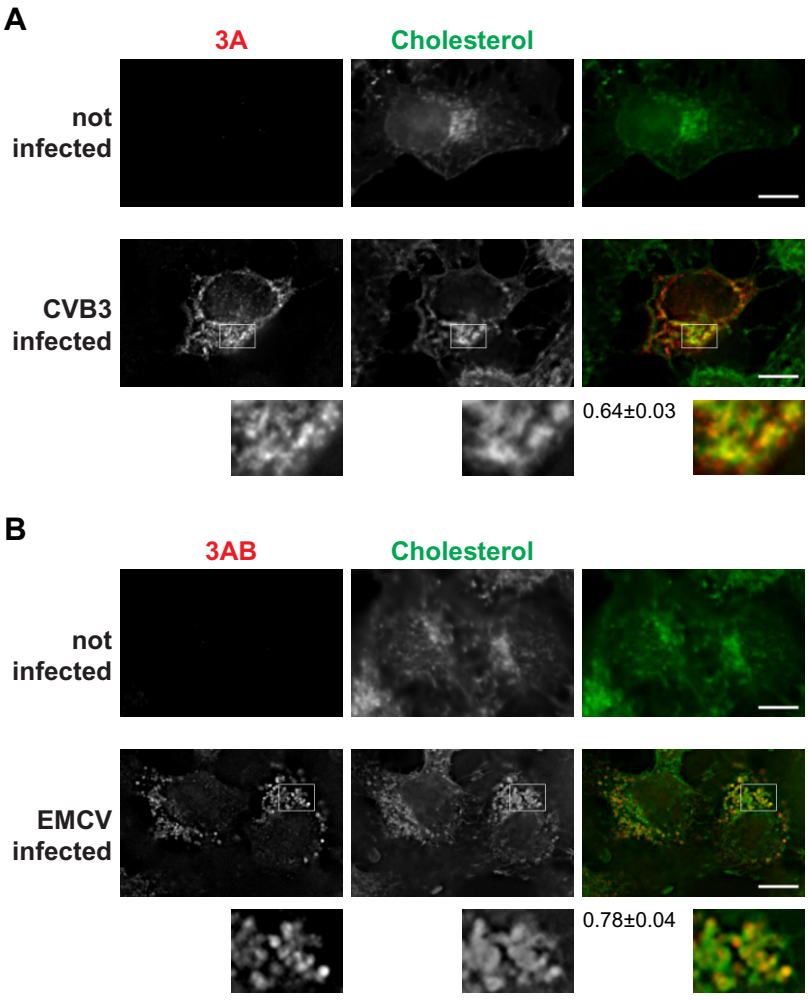


Fig. 1. Cholesterol is redistributed to replication organelles in CVB3- and EMCV-infected cells. HeLa cells infected with CVB3 wt (A) or EMCV wt (B) at a multiplicity of infection of 10 were fixed at 5 h post-infection (p.i.). Antibodies against CVB3 3A or EMCV 3AB were used for staining replication organelles and cholesterol was stained with filipin. The Manders' coefficients (fraction of 3A or 3AB structures containing cholesterol) for at least 10 infected cells are indicated. Scale bars correspond to 10 μm.

similar levels (~ 50%, Fig. 2C), the effects on CVB3 and EMCV replication were surprisingly different: MβCD inhibited CVB3 virus replication by ~ 70%, which is in agreement with the observations by Ilnytska *et al.* (2013), whereas HPCD reduced replication only by ~ 25%. Remarkably, MβCD did not reduce EMCV replication, while HPCD inhibited replication by ~ 90% (Fig. 2A and B). The inhibitory effects were specific and not due to any acute toxic effects of the treatments as revealed by a cell viability assay (Fig. 2D).

Cholesterol is important for CVB3 and EMCV genome replication

Depletion of cholesterol from the plasma membrane disturbs lipid rafts and this may affect the receptor distribution and, hence, virus entry. For instance, clustering of coxsackievirus and adenovirus receptor (i.e. the receptor for CVB3) is disrupted upon depleting cholesterol, resulting in decreased virus attachment and infectivity (Coyne and Bergelson, 2006; Wong *et al.*, 2006). Although we treated with the drugs only after infection, we set out to further demonstrate that the βCDs affected the replication stage. To this end, we performed a time of addition experiment in which we treated cells with cyclodextrin at different time points before and after infection. The cyclodextrin treatments had a similar profile as the established replication inhibitors guanidine HCl (CVB3) and dipyridamole (EMCV) and completely inhibited replication even when added at 2 h post-infection, indicating that the inhibitory effects of cholesterol depletion are unlikely due to defects in entry (Fig. 2E and F). Furthermore, we introduced replication-competent RNAs in the cells by transfection in order to omit the virus entry step. We used a CVB3 subgenomic replicon encoding Firefly luciferase (FLuc-CVB3) in place of the capsid-coding region or an EMCV genomic RNA encoding *Renilla* luciferase upstream of the leader sequence (RLuc-EMCV), and quantified replication by measuring luciferase production. We noted the same disparate effect of the two βCDs on the replication of

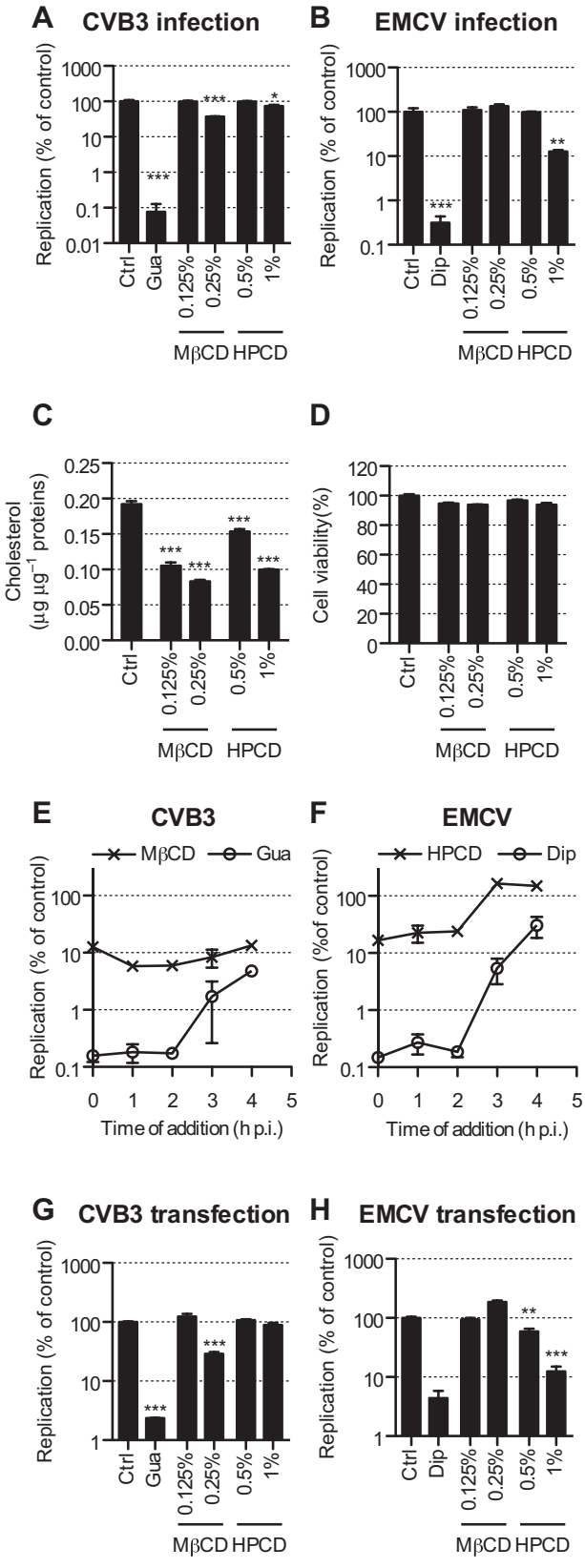


Fig. 2. Cholesterol depletion inhibits CVB3 and EMCV infection.
A and B. HeLa cells were infected with RLuc-CVB3 (A) or RLuc-EMCV (B) at a multiplicity of infection (MOI) of 0.5 and 1, respectively, for 30 min. After infection, fresh medium with 10% LPDS and the indicated concentrations of MβCD or HPCD, 2 mM guanidine HCl (Gua) (an inhibitor of CVB3 replication) or 100 μM dipyridamole (Dip) (an inhibitor of EMCV replication) was added. At 7 h p.i., cells were lysed and *Renilla* luciferase activity was determined.
C. Total cholesterol levels after depletion.
D. Cell viability assay performed in parallel.
E and F. HeLa cells were infected with RLuc-CVB3 (E) at an MOI of 0.1 or RLuc-EMCV at an MOI of 1 (F) and treated at the indicated time point after infection with MβCD 0.125% or Gua (E) and HPCD 1% or Dip (F) in medium with 10% LPDS. At 7 h p.i., cells were lysed and *Renilla* luciferase activity was determined.
G and H. HeLa cells were transfected with 5 ng FLuc-CVB3 replicon (G) or RLuc-EMCV RNA (H), treated as in (A and B) and lysed at 7 h post transfection (p.t.). Experiments were performed in triplicate and mean values ± SEM are shown. Treatments were statistically compared with the no drug control using the Student's *t*-test (A–C, G, H); $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

transfected RNAs (Fig. 2G and H) as on virus replication (Fig. 2A and B). Although we cannot exclude that the βCD treatments also have a small effect on virus entry, our data indicate that cholesterol is needed for the genome replication stage. These results show that, although CVB3 and EMCV depend on different host factors for replication, these viruses share a need for cholesterol for efficient genome replication. Because depletion of cholesterol with MβCD and HPCD to similar levels led to differential effects on CVB3 and EMCV replication, this implies that CVB3 and EMCV both rely on specific and different intracellular cholesterol pools.

Ongoing cholesterol synthesis is not important for CVB3 and EMCV replication

We also addressed the question whether ongoing cholesterol biosynthesis is important for virus replication. Cholesterol is synthesized in over 20 steps starting from acetyl-CoA (Ikonen, 2008). The rate-limiting enzyme of the pathway is 3-hydroxy-3-methylglutaryl-CoA reductase, which can be inhibited by a class of small molecules called statins. To investigate the involvement of ongoing cholesterol synthesis in virus replication, we inhibited cholesterol biosynthesis using lovastatin after virus infection (Fig. 3A and B). We performed the experiment in medium with LPDS instead of FCS to reduce the exogenous amount of cholesterol available for the cells, which leads to an up-regulation of cholesterol biosynthesis and an enhanced dependence of the cells on cholesterol biosynthesis. We found that lovastatin had no effect on virus replication when added during virus infection (Fig. 3A). Similar results were obtained using pravastatin (data not shown). This indicates that ongoing cholesterol

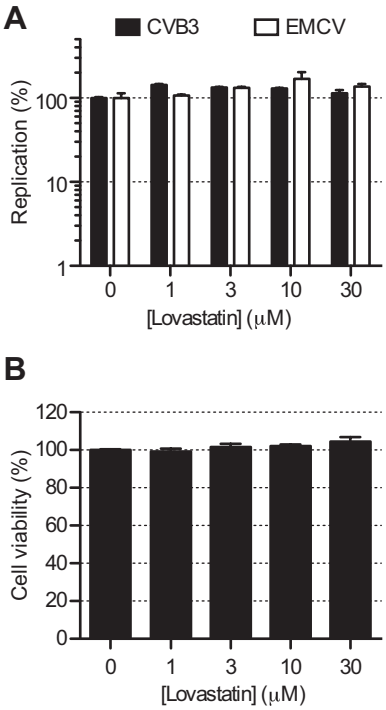


Fig. 3. Ongoing cholesterol biosynthesis is not required for CVB3 and EMCV replication. HeLa cells were infected with RLuc-CVB3 or RLuc-EMCV (A) as in Fig. 2. After infection, the cells were cultured in the presence of lovastatin in medium with 10% LPDS. At 7 h p.i., cells were lysed and *Renilla* luciferase activity was measured. B. A cell viability assay using MTS was performed in parallel. Experiments were performed in triplicate and mean values ± SEM are shown. Treatments were not statistically different from controls as analysed using the Student's *t*-test.

synthesis is not required for viral RNA replication. Apparently, the viruses rely mainly on pre-existing cholesterol and shuttle it to the ROs.

Drugs that affect endosomal cholesterol homeostasis inhibit virus replication

Next, we set out to study the involvement of endosomal cholesterol homeostasis in virus replication. Increased endosomal cholesterol content, which can be achieved by treating cells with amphipathic cationic amines such as U18666A, AY9944 or imipramine (Liscum and Faust, 1989; Cenedella, 2009), was reported to induce clustering of endosomes towards the negative end of the microtubules and the microtubule organizing centre (Mukherjee and Maxfield, 2004; Sugii *et al*., 2006). On the other hand, drugs that lower the endosomal cholesterol content such as cholesterol synthesis inhibitors belonging to the statin class (e.g. mevastatin and lovastatin) have the opposite effect and induce the dispersion of endosomes towards the plus end of the microtubules (Sugii *et al*., 2006; Rocha *et al*., 2009). To study the involvement of endosomal cholesterol homeostasis in virus replication, we treated cells with drugs that cause accumulation of endosomal cholesterol and the consequent clustering of endosomes (U18666A, AY9944 and imipramine) or that lower endosomal cholesterol and lead to their dispersion (mevastatin). We observed that the relocalization of cholesterol-laden endosomes upon U18666A treatment was rather slow and that the first changes were only observed after ~ 8 h of incubation, consistent with previous observations (Lloyd-Evans *et al*., 2008), but extensive clustering of cholesterol-containing endosomes was clearly visible after overnight treatment (not shown). Therefore, all drug treatments to disrupt endosomal cholesterol homeostasis were performed overnight. We tested several concentrations of the drugs and chose the lowest concentrations that yielded robust effects. We first investigated by filipin staining how the treatments affected the localization of cholesterol, which clearly colocalized with the endosomal marker LAMP1 [Pearson's colocalization coefficient (PCC) 0.73 in control-treated cells] (Fig. 4A). As expected, the cholesterol shuttling inhibitors led to an accumulation of cholesterol in the endosomes (Fig. 4A) (PCCs were somewhat elevated probably because these inhibitors prevent the redistribution of cholesterol out of endosomes). To quantify the effect of the drugs on cholesterol distribution, we determined the coefficient of variation, i.e. the standard deviation divided by the mean, of the filipin staining intensity as outlined in Fig. S1. In the case of an accumulation of cholesterol, the variation in staining intensity increases, which leads to an increase of the coefficient of variation. As expected, U18666A, AY9944 and imipramine caused a similar increase in the coefficient of variation, demonstrating the accumulation of cholesterol (Fig. 4A and B), whereas inhibition of cholesterol synthesis using mevastatin led to a dispersion of cholesterol (coefficient of variation significantly reduced) (Fig. 4A and B).

We then tested the effect of altered endosomal cholesterol homeostasis on CVB3 and EMCV replication. To bypass possible effects on virus entry and test directly whether U18666A, AY9944, imipramine, mevastatin or lovastatin affect genome replication, we introduced the FLuc-CVB3 replicon RNA or RLuc-EMCV genomic RNA in cells by transfection. In parallel, we transfected an RLuc mRNA to establish whether the treatment had any unspecific effects on RNA transfection or translation efficiency and measured cell viability and total cholesterol levels to exclude that the drugs inhibited replication by detrimental effects on cell viability or by depleting cellular cholesterol (Fig. 4E–G). We found that 10 μM U18666A completely inhibited the replication of CVB3 and EMCV (> 300-fold reduction of luciferase levels). This treatment, however, also reduced the transfection/translation efficiency of

RLuc mRNA by approximately 10-fold (Fig. 4C–E) and therefore it cannot be concluded that the almost complete abrogation of viral RNA replication is solely due to the effect of U18666A on endosomal cholesterol homeostasis. The other drugs, which either accumulate or disperse endosomal cholesterol, reduced CVB3 replication by 60–90% and EMCV replication by ~ 90% (Fig. 4C and D), without affecting reporter RLuc mRNA translation, cell viability or total cholesterol levels (Fig. 4E–G). We conclude that not the total cholesterol content but rather the endosomal cholesterol availability is important for replication.

Disrupting endosomal cholesterol homeostasis inhibits cholesterol recruitment to ROs

Next, we studied whether the drugs that disrupt endosomal cholesterol homeostasis affect the recruitment of cholesterol to ROs. To this end, we pretreated cells as above, infected them with CVB3 and stained with filipin to visualize cholesterol and with an antibody against the viral protein 3A as an RO marker. As before (Fig. 1), the colocalization of filipin with 3A shows that cholesterol accumulates at ROs. In particular U18666A, which strongly inhibits replication (Fig. 4C), severely reduced the recruitment of cholesterol to ROs (Fig. 5A and B). The other drugs, regardless of whether they cause a clustering or a scattering of endosomes (Fig. 4A and B), all inhibited the recruitment of cholesterol to ROs, albeit to a lesser extent than U18666A (Fig. 5A and B). Thus, any disruptions in endosomal cholesterol homeostasis, either cholesterol content or positioning, prevent the recruitment of cholesterol to ROs.

A CVB3 mutant that replicates independent of PI4KIIIβ depends on endosomal cholesterol homeostasis

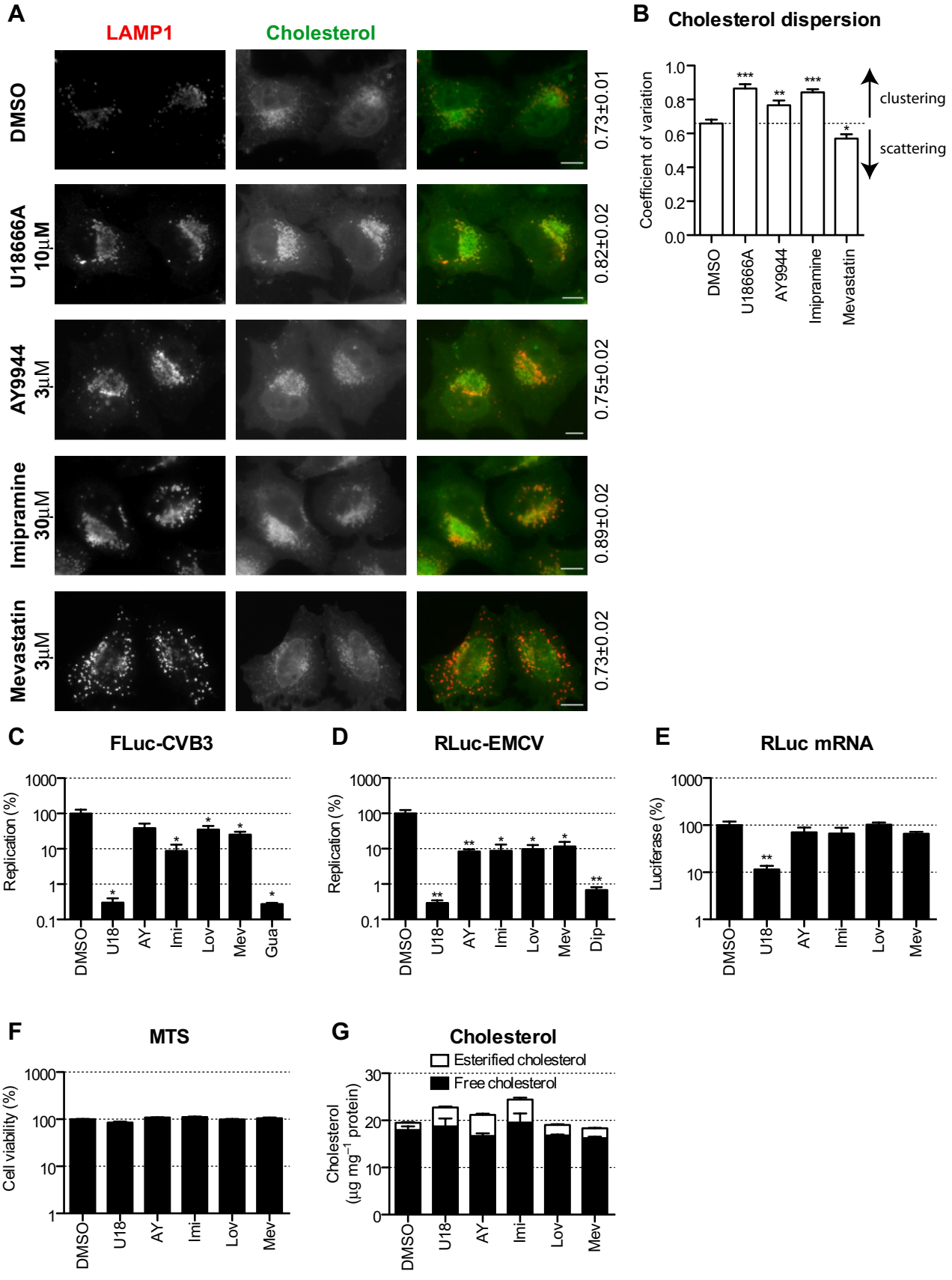
PI4KIIIβ was shown to be crucial for enterovirus replication, but single-point mutations in the enterovirus 3A protein can bypass the requirement for PI4KIIIβ (Arita *et al*., 2011; van der Schaar *et al*., 2012). The CVB3 3A protein was found to increase cholesterol levels at the ROs by recruiting cholesterol-rich recycling endosomes to ROs through an interaction between PI4KIIIβ and Rab11 (Ilnytska *et al*., 2013). We therefore addressed the question whether a mutation in 3A that allows PI4KIIIβ-independent replication (3A[H57Y]) also allows replication in the presence of drugs that disrupt endosomal cholesterol homeostasis. To this end, we compared the effects of the treatments used in Fig. 4 on wild-type (wt) replicon (FLuc-CVB3) and a replicon containing the H57Y mutation in 3A (FLuc-CVB3-3A[H57Y]). As expected, the 3A[H57Y] mutation allowed replication in the presence of the PI4KIIIβ inhibitor compound 1 (BF738735). However, the wt and 3A[H57Y] mutant replicons were equally inhibited by each of the treatments that affected endosomal cholesterol homeostasis (Fig. 6), indicating that the PI4KIIIβ-independence mutation does not protect against drugs that affect endosomal cholesterol. This suggests that the 3A[H57Y] mutation does not relieve the need of the virus for endosomal cholesterol for efficient replication, but may rather provide an alternative route to recruit cholesterol to ROs. It will remain a challenge to find out the alternative mechanism by which 3A[H57Y] recruits cholesterol independent of PI4KIIIβ.

Discussion

We here investigated the importance of cholesterol for the replication of CVB3 and EMCV, two picornaviruses from different genera. We for the first time show that EMCV, which relies on different host factors and membranes for replication than CVB3, recruited cholesterol to ROs similar to CVB3. We provide evidence that CVB3 and EMCV rely on different cholesterol pools for efficient replication and that endosomal cholesterol homeostasis is important for both viruses.

We found that CVB3 and EMCV both required cholesterol for efficient genome replication. However, CVB3 was inhibited stronger upon cholesterol depletion by MβCD than by HPCD, whereas EMCV was inhibited by HPCD only. Importantly, MβCD treatment was proposed to mainly depleted plasma membrane cholesterol (Keller and Simons, 1998), whereas HPCD is thought to also have a strong effect on ER cholesterol, depleting 80% of ER cholesterol, but only 25% of total membrane cholesterol in human primary fibroblasts (Lange *et al*., 1999). Thus, CVB3 and EMCV appear to rely on different pools of cholesterol for efficient replication. Importantly, EMCV has been suggested to replicate on ER-derived membranes (Gazina *et al*., 2002), which could explain why EMCV is more sensitive to HPCD than to MβCD. CVB3 was reported to recruit endosomal cholesterol (Ilnytska *et al*., 2013), which is in continuous exchange with the plasma membrane and highly sensitive to disruptions by MβCD.

We found that replication of CVB3 and EMCV depended not so much on the total amount of cholesterol in the cell, but rather on endosomal cholesterol homeostasis. Specifically, treatments that affect endosomal cholesterol levels and – as a result – the positioning of endosomes in the cell inhibited replication of both CVB3 and EMCV. Because endosomal positioning is dependent on cholesterol content (Rocha *et al*., 2009), we cannot distinguish whether virus replication is inhibited by altered cholesterol content or by changes in endosomal positioning. The finding that compounds that either increase or decrease endosomal cholesterol


A
LAMP1
Cholesterol
DMSO
U18666A 10μM
AY9944 3μM
Imipramine 30μM
Mevastatin 3μM
0.73±0.01
0.82±0.02
0.75±0.02
0.89±0.02
0.73±0.02
B
Cholesterol dispersion
Coefficient of variation
clustering
scattering
DMSO
U18666A
AY9944
Imipramine
Mevastatin
C
FLuc-CVB3
Replication (%)
DMSO
U18
AY
Imi
Lov
Mev
Gua
D
RLuc-EMCV
Replication (%)
DMSO
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Mev
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Cell viability (%)
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Imi
Lov
Mev
G
Cholesterol
Esterified cholesterol
Free cholesterol
Cholesterol (μg mg−1 protein)
DMSO
U18
AY
Imi
Lov
Mev

Fig. 4. Endosomal cholesterol is important for replication.
A. HeLa cells were treated for 23 h with the indicated compounds, fixed and stained for cholesterol with filipin and against the endosomal marker LAMP-1. Pearson's coefficient of colocalization for at least 10 cells for each condition is indicated.
B. Cholesterol dispersion was measured (as described in Fig. S1) in at least 10 cells per condition treated as in (A).
C–G. HeLa cells were pretreated for 16 h with drugs that affect endosomal cholesterol homeostasis [0.1% DMSO, 10 μM U18666A (U18), 3 μM AY9944 (AY), 30 μM imipramine (Imi), 3 μM Lovastatin (Lov), 3 μM Mevastatin (Mev)]. Then, cells were transfected with 5 ng of FLuc-CVB3 replicon RNA (C) or RLuc-EMCV RNA (D), or 30 ng of RLuc mRNA (E) for 30 min, fresh medium containing above-mentioned drugs, 2 mM Gua or 100 μM Dip was added, cells were lysed at 7 h p.t., and luciferase activity was measured. In parallel, a cell viability assay was performed (F) and the levels of free and esterified cholesterol were measured using Amplex Red cholesterol quantification kit (G). Scale bars correspond to 10 μm. Replication experiments were performed in triplicate. Mean values ± SEM are shown. Treatments were statistically compared with the DMSO control using the Mann–Whitney test (B) or the Student's *t*-test (C–E); $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

content have similar inhibitory effects on virus replication suggests that the endosomal cholesterol level *per se* is not important for replication. Instead, endosomes are directed to either end of the microtubules by the treatments and may therefore not be efficiently recruited by the viruses and used to provide cholesterol to the ROs. The cholesterol shuttling inhibitors that we used accumulate cholesterol in late endosomal compartments. At first glance, our data seem to disagree with the finding that CVB3 recruited cholesterol from recycling endosomes (Ilnytska *et al*., 2013). However, shuttling inhibitors prevent recycling of cholesterol from late endosomal compartments to, among others, the plasma membrane and thus will also affect recycling endosomal cholesterol. In addition, alterations of cholesterol homeostasis could affect the endosomal system in other ways. Namely, cholesterol depletion induces the dissociation of Rab11 from the membranes of the recycling endosomes into the cytoplasm (Takahashi *et al*., 2007). This implies that cholesterol depletion could hamper CVB3 replication by preventing the recruitment of Rab11-containing endosomes to ROs either by redistributing recycling endosomes or by decreasing the Rab11 amount on the membrane. Alternatively, the treatments may all have a similar effect on recycling endosomes, which are the type of endosomes involved in delivering cholesterol to CVB3 ROs (Ilnytska *et al*., 2013). Namely, the shuttling inhibitors accumulate cholesterol mainly in late endosomes/lysosomes and prevent the recycling of cholesterol via recycling endosomes, thus lowering the cholesterol content of recycling endosomes, while statins reduce the cholesterol content of all endosomes, including recycling endosomes. In both cases, recycling endosomes contain lower cholesterol levels and delivery of cholesterol to ROs via recycling endosomes is impaired. Consistently, the impairment of endosomal cholesterol homeostasis reduced the recruitment of cholesterol to ROs by CVB3, which explains why these drugs inhibit virus replication. Importantly, previous studies on the importance of cholesterol for CVB3 replication (Ilnytska *et al*., 2013) may also have detected primarily the importance of the endosomal cholesterol pool as extraction of cholesterol using cyclodextrins also affects endosomal positioning and motility (Chen *et al*., 2008).

The results from depleting cholesterol with cyclodextrins suggested that EMCV relies on a different cholesterol pool than CVB3, possibly an ER pool. Nevertheless, EMCV was also sensitive to disruptions of endosomal cholesterol. This may be explained by the tight contacts between endosomes and the ER (Rocha *et al*., 2009; Eden *et al*., 2010; West *et al*., 2011) that are thought to allow the exchange of cholesterol between these organelles (Rocha *et al*., 2009). Thus, by affecting endosomal cholesterol homeostasis we have likely affected ER cholesterol as well.

One of the compounds we used to alter endosomal cholesterol homeostasis, U18666A, is commonly used to induce a phenotype similar to Niemann-Pick type C disease (NPC) in terms of cholesterol localization. This disease is caused by loss-of-function mutations in endosomal cholesterol transporter proteins, preventing cholesterol export out of endosomes. Enterovirus replication was shown to be stimulated in primary cells from individuals with NPC (Ilnytska *et al*., 2013). However, we show that U18666A inhibits virus replication. The apparent discrepancy may lie in the fact that U18666A has two effects on cholesterol: it induces cholesterol accumulation in the endosomes and it inhibits ER cholesterol biosynthesis, which in our experiments balanced each other, as we did not see a change in free cholesterol levels. In NPC–/– cells, cholesterol synthesis is not affected, leading to a strong increase in free cholesterol levels, which is beneficial to virus replication. Furthermore, our findings demonstrate that U18666A and other cationic amphiphiles do not mimic the NPC phenotype in all ways and that care should be taken when using these drugs to mimic an NPC phenotype.

Summarizing, we show for the first time that EMCV recruits and requires cholesterol, and that both CVB3 and EMCV are sensitive to disruptions of cholesterol shuttling through the endosomal compartment. Our results show that cholesterol has a significant role in genome replication of both CVB3 and EMCV, members of the enterovirus and cardiovirus genera respectively.

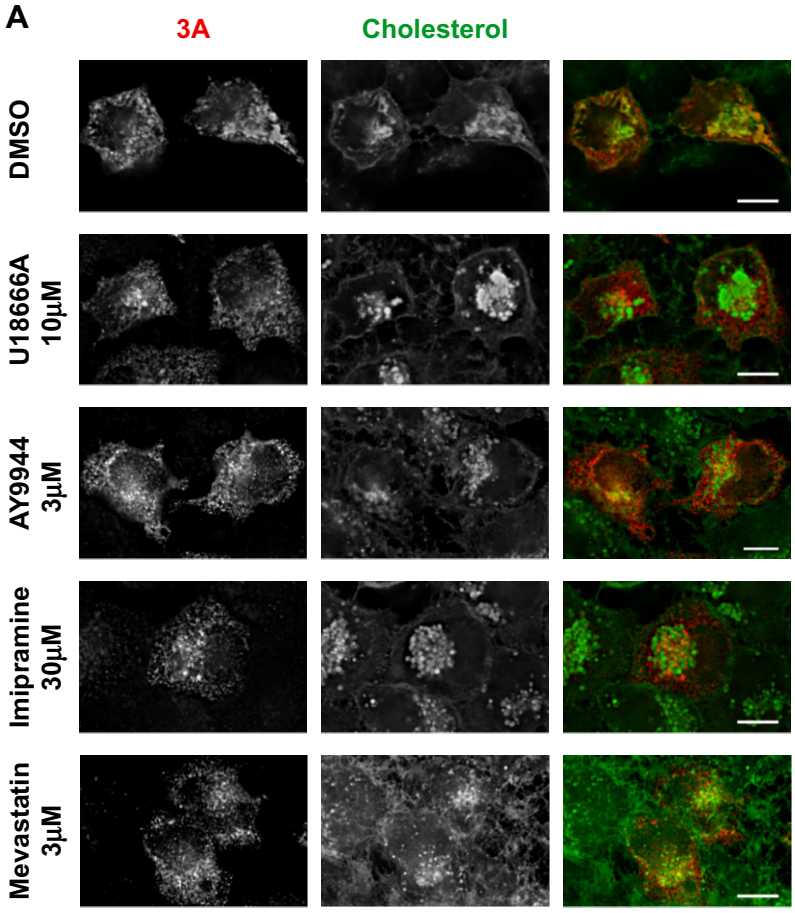

Fig. 5. Drugs that affect endosomal cholesterol homeostasis inhibit cholesterol recruitment to CVB3 ROs.
A. HeLa cells were pretreated with compounds as in Fig. 4 and infected with CVB3 at a multiplicity of infection of 10 for 30 min after which fresh, compound-containing medium was added and cells were fixed at 5 h p.i. and stained as in Fig. 1.
B. Manders' coefficient for 3A structures containing cholesterol was calculated for at least 10 infected cells per condition. Scale bars correspond to 10 μm. Shown are means ± SEM. Treatments were statistically compared with the DMSO control using the Mann–Whitney test; $*P < 0.05$, $***P < 0.001$.

Because picornaviruses from the same genus utilize highly similar replication strategies and depend on the same set of host factors, other enteroviruses and cardioviruses may also require cholesterol for their genome replication. Besides CVB3, the enteroviruses poliovirus, rhinovirus 2 and echovirus 11 were shown to recruit cholesterol to ROs (Ilnytska *et al.*, 2013). On the other hand, infectivity of the enterovirus echovirus 1 was reported not to depend on cholesterol (Siljamäki *et al.*, 2013). However, these studies applied different strategies to disrupt cholesterol and to assess virus replication and may therefore not be directly comparable. Future

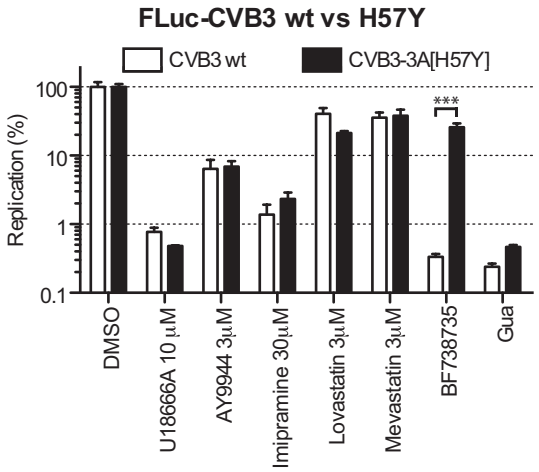

Fig. 6. CVB3-3A[H57Y] mutant is inhibited by drugs affecting endosomal cholesterol. HeLa cells were pretreated with compounds as in Fig. 4 and transfected with either FLuc-CVB3 replicon or CVB3 3A[H57Y]. The PI4KIIIβ inhibitor compound 1 (van der Schaar *et al*., 2013) and guanidine were only added after transfection, not during the pretreatment. Controls for RLuc mRNA translation, cholesterol quantification, cell viability and cholesterol redistribution as in Fig. 4C–F were performed in parallel and were in agreement with the observations in Fig. 4 (not shown). Experiments were performed in triplicate and mean values ± SEM are shown. For each treatment, wt and 3A[H57Y] were statistically compared using the Student's *t*-test, ***$P < 0.001$, only differences < 0.01 are indicated.

studies should resolve whether all enteroviruses require cholesterol for replication or whether there are fundamental differences between different members of this genus. We show that not the absolute amount of cholesterol in the cell but rather endosomal cholesterol homeostasis is important for virus replication. Although our work clearly shows the importance of endosomal cholesterol, these viruses may also exploit other sources of cholesterol. For example, rhinoviruses were recently suggested to mobilize cholesterol from a storage pool in lipid droplets and distributed it to ROs via the ER, involving a number of cholesterol-shuttling proteins from the family of oxysterol-binding protein (OSBP)-related proteins (Roulin *et al*., 2014). Shuttling of cholesterol to ROs by OSBP has also been shown to occur in poliovirus- and coxsackievirus-infected cells (Arita *et al*., 2013; Arita, 2014; Wang *et al*., 2014; Strating *et al*., 2015), and it is therefore likely that CVB3 and other enteroviruses use this mechanism to create cholesterol-rich ROs as well.

It remains to be shown what the role of cholesterol at the ROs is and whether these viruses use other cholesterol sources besides endosomal cholesterol. Cholesterol is important for membrane properties, such as fluidity/rigidity, permeability and the formation of lipid rafts. Interestingly, cholesterol appears to regulate the formation of microdomains enriched in phosphoinositides including PI4P and may enhance the binding of proteins to phosphoinositides (Jiang *et al*., 2014). Therefore, cholesterol may organize PI4P domains at the ROs and enhance the recruitment of PI4P-binding proteins.

Experimental procedures

Cells and reagents

HeLa R19 cells were grown in Dulbecco's minimal essential medium (Lonza) supplemented with 10% foetal bovine serum at 37°C and 5% CO_2. MβCD, hydroxypropyl-β-cyclodextrin, imipramine, lovastatin and mevastatin were purchased from Sigma-Aldrich; U18666A was purchased from Cayman Chemical; and AY9944 was from Tocris Bioscience.

Virus infection and replicon assays

RLuc-CVB3 virus was generated from plasmid pRLuc-CVB3/T7(-3A[H57Y]) described previously (Wessels *et al*., 2005) and produced in BGM cells. The subgenomic replicon pRib-LUC-CB3/T7 was described previously (Lanke *et al*., 2009). The 3A[H57Y] mutation was inserted into this plasmid by replacing the wt sequence with an insert containing the mutation excised from p53CB3/T3-3A[H57Y] (De Palma *et al*., 2009) using Bst1107I and BlnI. RLuc-EMCV was derived from the clone pRLuc-QG-M16.1. This plasmid contains a cDNA copy of EMCV, strain mengovirus, with an open reading frame that encodes the first six amino acids of the leader protein (L), *Renilla* luciferase, GlnGly (for cleavage by the viral 3C protease) followed by the viral polyprotein (without the first Met and amino acids 2–6 of the leader protein encoded by alternative codons to prevent recombination). The plasmid was constructed as follows: first, a multiple cloning site (XhoI, NheI, SacI/EcoICRI, NotI), codons for the amino acids GlnGly, and changed codons for amino acids 2–6 of the leader were introduced into pM16.1(XhoI/HpaI) (Hato *et al*., 2010) to generate pM16-MCS-QG-L26. Next, the *Renilla* luciferase-coding region from pBind (Promega) was introduced in the vector pM16-MCS-QG-L26. Linearization of the infectious clone for *in vitro* transcription was performed with BamHI and RNA was transfected using Lipofectamine 2000 (Invitrogen). RLuc-EMCV was produced in BHK cells.

Virus infections were performed at 37°C for 30 min, after which the virus was removed and medium with compounds was added [t = 0; all indicated time points post-infection (p.i.) are counted from t = 0]. In the time-of-addition experiments, medium without compound was added at t = 0, which was replaced by medium with compound at the indicated time points. Virus replication was assessed by measuring Firefly or *Renilla* luciferase activity using a Firefly or *Renilla* luciferase assay system (Promega).

Cholesterol quantification

Free and esterified cholesterol was determined enzymatically using the Amplex Red kit (Invitrogen). Samples were normalized to equal amounts of protein as determined by BCA assay (Thermo Scientific).

Immunofluorescence microscopy

HeLa R19 cells were grown to subconfluency on coverslips in 24-well plates. Where indicated, cells were treated with compounds or infected with CVB3 or EMCV at a multiplicity of infection of 10 at 37°C for 30 min, after which the virus was removed and medium with compounds was added (t = 0). Cells were fixed with 4% paraformaldehyde for 20 min at room temperature at the indicated time point followed by permeabilization with 0.2% saponin/0.5% BSA in PBS for 10 min. Cells were then incubated sequentially with primary and secondary antibodies diluted in PBS containing 2% normal goat serum. Viral CVB3 3A was detected using a mouse monoclonal antibody described before (Dorobantu *et al*., 2014); EMCV 3AB was detected using a mouse monoclonal antibody provided by Aminev *et al*. (2003), and the mouse anti-LAMP-1 antibody (H4A3) was from BioLegend. Conjugated goat-anti-mouse Alexa Fluor 594 (Molecular Probes) was used as secondary antibody. Cholesterol was stained with 25 $\mu g\ ml^{-1}$ filipin III (Sigma) (diluted from a 25 $mg\ ml^{-1}$ stock in DMSO) for 1 h at room temperature included during the incubation with the secondary antibody. Coverslips were mounted with FluorSave (Calbiochem), and images were acquired with a Nikon Eclipse Ti microscope equipped with a high-sensitivity EM-CCD camera (Andor DU-897) or sCMOS camera (Andor Zyla). Filipin images were acquired in wide-field mode using a 405/20 excitation filter, 425LP dichroic mirror and 460/50 emission filter. Alexa Fluor 594 was imaged using a 545/25 excitation filter, 565LP dichroic mirror and 605/70 emission filter. Images were deconvoluted performing 20 iterations using NIS-Elements software (Nikon). The deconvoluted images were used to analyse colocalization between cholesterol and ROs using ImageJ as follows. Single-infected cells were outlined and signal from outside the cell was excluded from calculations. MCC for at least 10 cells per condition was calculated using the JACoP plug-in for ImageJ (Bolte and Cordelieres, 2006). Because the Costes' automatic thresholding values were too low, manually set thresholds were used in order to prevent background fluorescence and fluorescence from diffuse staining to affect the analysis. PCC for at least 10 cells for each condition was calculated using the Coloc2 plug-in for ImageJ with default settings. To quantify the effects on cholesterol distribution, at least 10 cells for each condition were analysed using an ImageJ script performing the following steps as outlined in Fig. S1: generate a $3 \times 3\ \mu m$ grid on top of the single cells, followed by measuring the mean filipin intensity in the individual grid boxes obtained and finally calculating the coefficient of variation for all the boxes for each cell.

Statistics

Unpaired one-tailed Student's *t*-test or two-tailed Mann–Whitney test were used to compare the control and experimental groups as indicated in the figure legends.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Quantification of cholesterol distribution. Workflow to quantify the distribution of cholesterol in cells treated with drugs that affect cholesterol distribution (Fig. 4A). Cells were treated with drugs, fixed and stained for cholesterol using filipin. Images were processed as follows: background was subtracted from the raw images and single cells were selected. For each cell, a 3×3 μm grid was overlaid and the mean fluorescence intensity in each box of the grid was calculated. For each cell, the mean and standard deviation of all boxes of the grid were calculated and the coefficient of variation (i.e. standard deviation divided by mean) was calculated as a measure for the distribution of cholesterol.