Exosome mimicry by a HAVCR1-NPC1 pathway of endosomal fusion mediates hepatitis A virus infection

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Cell-to-cell communication by exosomes controls normal and pathogenic processes\(^{4,5}\). Viruses can spread in exosomes and thereby avoid immune recognition\(^2\). While biogenesis, binding and uptake of exosomes are well characterized\(^{4,5}\), delivery of exosome cargo into the cytoplasm is poorly understood\(^6\). We report that the phosphatidylserine receptor HAVCR1 (refs. 6,7) and the cholesterol transporter NPC1 (ref. 8) participate in cargo delivery from exosomes of hepatitis A virus (HAV)-infected cells (exo-HAV) by clathrin-mediated endocytosis. Using CRISPR-Cas9 knockout technology, we show that these two lipid receptors, which interact in the late endosome\(^9\), are necessary for the membrane fusion and delivery of RNA from exo-HAV into the cytoplasm. The HAVCR1-NPC1 pathway, which Ebola virus exploits to infect cells\(^9\), mediates HAV infection by exo-HAV, which indicates that viral infection via this exosome mimicry mechanism does not require an envelope glycoprotein. The capsid-free viral RNA in the exosome lumen, but not the endosomal uncoating of HAV particles contained in the exosomes, is mainly responsible for exo-HAV infectivity as assessed by methylene blue inactivation of non-encapsidated RNA. In contrast to exo-HAV, infectivity of HAV particles is pH-independent and requires HAVCR1 or another as yet unidentified receptor(s) but not NPC1. Our findings show that envelope-glycoprotein-independent fusion mechanisms are shared by exosomes and viruses, and call for a reassessment of the role of envelope glycoproteins in infection.

Extracellular vesicles are heterogeneous cargo-containing vesicles secreted by cells that mediate intercellular communication. Extracellular vesicles include microvesicles, which are approximately 50–1,000 nm in diameter and bud from the plasma membrane, and exosomes, which are approximately 50–150 nm in diameter and produced in endosomal compartments\(^6,10\). Virus-infected cells secrete exosomes containing viral proteins, virus particles, nucleoproteins and capsid-free genomes that mediate virus spread and pathogenesis while evading immune recognition\(^1\). Hepatitis A virus (HAV), a non-enveloped positive-sense RNA Picornaviridae that causes acute hepatitis in humans\(^11\), presents a unique model to study cargo delivery because it establishes persistent infections in cell culture that produce significant amounts of easily purifiable exosomes containing viral RNA and viral particles in the exosome lumen\(^2\), which could be used as markers of cargo delivery. Exosomes from HAV-infected cells have been extensively characterized\(^11\), and we use similar reagents and conditions to produce exosomes in this work. Feng et al.\(^11\) termed the exosomes and viral particles purified from HAV-infected cells as “enveloped HAV” and “naked HAV”, respectively. However, we find this nomenclature misleading and use the terms “exosomes from HAV-infected cells (exo-HAV)” instead of enveloped HAV and “viral particle HAV (vphHAV)” instead of naked HAV because HAV is a non-enveloped virus. Moreover, the exosomes produced in HAV-infected cells are bona fide exosomes\(^6\) that contain viral particles and genomes, as described for a wide variety of other viruses\(^1\). After binding to the cell surface, exosomes can trigger cell signalling events, fuse at the cell surface and/or be internalized via endocytic pathways to deliver their cargo into recipient cells via the transfer of components such as lipids, membrane-bound proteins and lumen content, including coding and noncoding RNAs\(^1\). Binding and uptake of exosomes has been extensively studied, but the mechanisms involved in the delivery of lumen cargo into the cytoplasm remain poorly understood. Phagocytic cells take up exosomes via a process that is independent of HAVCR1 but requires TIM4, a phosphatidylserine (PS) receptor of the same family, which results in cargo degradation\(^1\). Other cell types use alternative pathways such as clathrin-mediated endocytosis (CME) and micropinocytosis to take up exosomes and deliver their cargo at the late endosome (LE)\(^9\) in a process that avoids degradation in endolysosomes. Some viruses, such as Ebola virus (EBOV) and Lassa virus, uncoat their genomes at LE compartments for productive infection\(^1\). However, the mechanisms and host proteins involved in exosome cargo delivery and endosomal uncoating of viruses are far from understood. Here, we studied how exosomes deliver functional mRNA from the lumen cargo into the cytoplasm. Viruses that uncoat their genomes in the LE probably share a similar mechanism that we term “exosome mimicry”.

Exosomes lack viral envelope glycoproteins (GPs) that mediate membrane fusion; therefore, we investigated whether receptors responsible for binding exosomes to the cell surface are also involved in fusion of the exosome and endosomal-delimiting membranes. A plethora of receptors, including integrins, lectins, PS receptors and heparan sulfate proteoglycans, mediate binding of exosomes to the cell surface\(^1\). We focused our attention on HAVCR1 (refs. 6,7), a membrane-bound PS receptor that mediates phagocytosis of apoptotic cells\(^1\), because PS is enriched on the outer leaflet of the exosome-delimiting membranes\(^4,14\) and HAVCR1 functions as a virus receptor\(^6,16-21\). To study the role of HAVCR1 in fusion, we transfected HAVCR1 complementary DNA into human embryonic kidney HEK-293 cells, which resulted in the expression of functional HAVCR1 at the cell surface (Fig. 1a; Supplementary Fig. 1). We analysed fusion using liposomes containing equal amounts of PS, phosphatidylcholine (PC) and cholesterol (Chl) labelled with

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octadecyl rhodamine B chloride (R18) (PS:PC:Chl–R18 liposomes), an autoquenched membrane dye that fluoresces after fusion with cell membranes. The PS:PC:Chl–R18 liposomes rapidly fused with membranes in HAVCR1 transfectants (Fig. 1b), whereas fusion was not detected in vector-transfected cells. In human hepatoma Huh7 cells, which naturally express HAVCR1 at the cell surface and
are susceptible to HAV infection, fusion of the PS:PC:Chl–R18 liposomes was temperature dependent, occurring mainly at 37°C compared with 4°C and 15°C (Extended Data Fig. 1a,b). Fusion of liposomes was also dependent on the presence of Chl and PS on the liposomes (Extended Data Fig. 1c,d) and on low intracellular pH, since treatment of cells with the pH inhibitors chloroquine, monensin or ammonium chloride (NH₄Cl) inhibited fusion (Extended Data Fig. 2a–c). Fusion occurred at intracellular membranes and not at the cell surface (Extended Data Fig. 2d), which indicates that HAVCR1 forms part of a cellular pathway that mediates endosome fusion. This result is in disagreement with previous findings. To determine whether HAVCR1 can mediate cargo delivery of functional RNAs from the lumen of exosomes to the cytoplasm, we analysed the mechanism of infection of exo-HAV, which contain PS at the surface (Extended Data Fig. 3a), and HAV-free RNA and viral particles in the lumen that serve as markers of cargo delivery. Purified exo-HAV and vPHAV were obtained by isopycnic gradient centrifugation from supernatants of cells infected with wild-type HAV containing a blasticidin (Bsd)-selectable marker (HAVBY–Bsd) (Fig. 1c, left panel), which confers Bsd resistance to infected cells. Characterization of exo-HAV according to the recommendations of the International Society for Extracellular Vesicles showed enrichment of the following proteins: (1) acetylcholinesterase (AchE) (Fig. 1c, right panel); (2) the chaperone 70-kDa heat shock protein (HSP70), the lipid raft marker flotillin-1 (FLOT-1) and the biogenesis factor tumour susceptibility gene 101 protein (TSG101), but not Golgi-specific golgin subfamily A member 1 (GOLGA1) (Extended Data Fig. 3b,c); and (3) the tetraspanins CD63 and CD81, but not CD9 at the exo-HAV surface (Extended Data Fig. 3d–f). They also exhibited a particle size of 166±6.4 nm (Extended Data Fig. 3g). These are all characteristics of bona fide exosomes and further support the notion that exo-HAV are exosomes containing HAV. Both exo-HAV and vPHAV showed similar specific infectivity in Huh7 cells (Extended Data Fig. 4a). Infectivity of exo-HAV (Fig. 1d, upper panel) but not vPHAV (lower panel) was blocked by liposomes containing PS:PC:Chl and PS:PC, but not PC:Chl and PC, which indicates that endocytosis of liposomes and exo-HAV share a common pathway. Huh7 HAVCR1 knockout (HAVCR1 KO) cells, which lack HAVCR1 expression (Fig. 1e), were resistant to exo-HAV infection compared with parental Huh7 cells, as assessed by the number of Bsd-resistance colony forming units (CFUs) (Fig. 1f, upper panels). Control vPHAV infected both parental and HAVCR1 KO cells (Fig. 1f, lower panels), which indicates that Huh7 cells express an alternative as yet unidentified HAV receptor specific for HAV particles but not exosomes. Huh7 HAVCR1 KO cells transfected with HAVCR1 cDNA expressed HAVCR1 at the cell surface (Fig. 1g, upper panel) and regained susceptibility to exo-HAV (Fig. 1h; Extended Data Fig. 4b). Transfectants expressing HAVCR1 containing a N94A mutation (Fig. 1g, lower panel), which abrogates PS receptor function, failed to regain exo-HAV cargo delivery function (Fig. 1h; Extended Data Fig. 4b). Taken together, these data further confirm the role of the HAVCR1 pathway in exosome cargo delivery.

We hypothesized that exo-HAV-mediated infection could be due to two different mechanisms: (1) degradation of the exo-HAV-delimiting membrane by lipases and endosomal uncoating of vPHAV by intracellular receptors or (2) fusion of the exo-HAV and endosome-limiting membranes and delivery of the capsid-free HAV genomes into the cytoplasm. To test our hypothesis, we analysed the role of the capsid-free HAV genome cargo in exo-HAV infection. Treatment of exo-HAV with detergent and RNase before sedimentation through a 40% sucrose cushion revealed that the exo-HAV cargo contained approximately 90% capsid-free HAV RNA (RNase sensitive), which migrated into the cushion, and 10% vPHAV (RNase resistant), which was pelleted through the sucrose cushion (Fig. 2a). The free RNA was protected within the exosome lumen since treatment with propidium monoazide (PMA), an impermeant nucleic-acid-intercalating photoreactive dye that blocks quantitative PCR with reverse transcription (RT–qPCR), did not affect nucleic acid amplification except after a heat shock (Fig. 2b). Treatment with methylene blue (MB), a photoreactive dye that penetrates membranes but not viral capsids, significantly reduced the infectivity of exo-HAV but not vPHAV (Fig. 2c). Huh7 HAVCR1 KO transfectants expressing the chimeric receptor HAVCR1ΔIgV/ANX5 (Fig. 2d; Extended Data Fig. 4c), in which the immunoglobulin variable (IgV)-like domain of HAVCR1 that interacts with PS and vPHAV is replaced with annexin V (ANX5; a PS but not a vPHAV receptor), bound apoptotic cells (Extended Data Fig. 4d) and regained susceptibility to exo-HAV infection (Extended Data Fig. 4e). Furthermore, African green monkey kidney (AGMK) HAVCR1 KO cells, which are resistant to both exo-HAV and vPHAV infection, transfected with HAVCR1ΔIgV/ANX5 cDNA expressed this chimeric receptor at the cell surface (Fig. 2e) and regained susceptibility to exo-HAV but not vPHAV (Fig. 2f) purified from supernatants of AGMK cells infected with HAV PI (Fig. 2g), a virus derived from infectious cDNA and passaged 100 times in cell culture. Taken together, these data show that the HAVCR1 pathway mediates cargo delivery of free RNA from the lumen of exosomes into the cytoplasm, and that endosomal uncoating of vPHAV is not required for exo-HAV-mediated infection.

HAVCR1 colocalizes in the LE with NPC1 (ref. 30), a cholesterol transporter enriched in the LE, which suggests that their interaction is important for exosome cargo delivery. To evaluate the role of NPC1 in exosome cargo delivery, we used NPC1 KO cells that are resistant to EBOV infection (Extended Data Fig. 5a) but not to other enveloped viruses that also require endosomal acidification for infection. Huh7 NPC1 KO cells expressed HAVCR1 at the cell surface (Fig. 3a) and bound apoptotic cells (Fig. 3b), which indicates that the HAVCR1 PS receptor function was not affected by NPC1 KO. PS:PC:Chl–R18 liposomes fused at the endosomes of Huh7 parental but not NPC1 KO cells (Fig. 3c; Extended Data Fig. 5b,c), which indicates that NPC1 KO prevented endosomal fusion. Consistent with this finding, Huh7 NPC1 KO cells did not support exo-HAV-mediated infection, but were permissive for vPHAV infection (Fig. 3d,e), which shows that NPC1 is required for exosome RNA cargo delivery but not HAV replication. Because EBOV requires NPC1 (refs. 24,29) to infect cells, we analysed whether exo-HAV and exosomes from uninfected cells (Fig. 4a) shared their cell entry pathway with EBOV. Exosomes and exo-HAV from uninfected cells blocked the cell entry of vesicular stomatitis virus (VSV) pseudotyped with EBOV GP but not VSV infection (Fig. 4b), which does not require NPC1, thus suggesting that exosomes, exo-HAV and EBOV share a common cell entry mechanism that requires NPC1. A bimolecular fluorescence complementation (BiFC) assay of the HAVCR1–NPC1 interaction based on monomeric Kusabira green reporter fluorescence protein (Extended Data Fig. 6) revealed that the following mutations did not affect the expression of NPC1 and its interaction with HAVCR1 at the LE: double mutations in the Chl-binding pocket at the amino-terminal domain (L175A/L176A and P202A/F203A) that prevents NPC1 function; a mutation in the carboxyl domain (F503Y) that reduces EBOV infectivity by 2 logs and a mutation in the same residue (F503W) that enhances EBOV GP binding; mutations in transmembrane domains (G660R and P691S) that affect NPC1 function; and a mutation in the sterol-sensing domain (L656F) that increases Chl binding. However, all these NPC1 mutations affected the infection of exo-HAV (Extended Data Fig. 7a) and the cell entry of EBOV, except for F503W, which did not affect the latter (Extended Data Fig. 7b). Taken together, these results indicate that EBOV and exo-HAV use similar cell entry pathways to infect cells.

Liposome fusion (Extended Data Fig. 2a–c) and cell entry of exo-HAV but not vPHAV are pH-dependent endocytic mechanisms.
To further understand the exo-HAV cargo delivery pathway, we used inhibitors of endocytosis \(^{14}\) to block exo-HAV infectivity (Fig. 4c). Treatment with chlorpromazine (CPZ), which specifically blocks CME, or dynasore, which blocks both CME and caveolae-mediated endocytosis and affects the early endosome (EE) compartment \(^{14}\), blocked entry of exo-HAV. However, treatment with 5-(N-ethyl-N-isopropyl) amiloride (EIPA) or LY294002, both of which block micropinocytosis and phagocytosis, did not prevent the cell entry of exo-HAV. Interestingly, the EE-to-LE transport blocker 4-bromobenzaldehyde \(N\)-(2,6-dimethylphenyl)
semicarbazone (EGA) also prevented exo-HAV cargo delivery. It should be pointed out that treatment with CPZ, dynasore or EGA did not affect cell viability (Extended Data Fig. 7c), and that EIPA and LY924002 were active, as assessed by their inhibition of EBOV cell entry (Extended Data Fig. 7d). Infection of exo-HAV and vpHAV share the same endocytic pathway (Extended Data Fig. 7e), but with...
Fig. 4 | Exosome cell entry by CME and cargo delivery via the HAVCR1-NPC1 pathway. a, Exosomes purified from cell culture supernatants of mock-infected or HAV-infected cells by isopycnic ultracentrifugation and peaks identified by AcH assay. Data are representative of three independent experiments. b, Blocking of EBOV (rVSV-EBOVgp–GFP) and VSV (VSV–GFP) cell entry by treatment with purified exosomes from uninfected cells or exo-HAV from a. GFP fluorescence was determined at 16-20h post-treatment compared with media-treated cells. Data are shown as the mean±s.e.m., n=6 from 3 independent experiments with 2 biological replicates. P values were determined by unpaired, two-sided Mann–Whitney test. c, Block of exo-HAV cargo delivery by cell entry inhibitors. HAV RNA genomes were quantitated by RT-qPCR at 72h post-treatment. Data are shown as the mean±s.e.m; from left to right, n=5, 5, 3, 3 and 3. P values between DMSO and inhibitors were determined by unpaired, one-way ANOVA with Dunnett’s post-test. 

The main difference that vpHAV infection is pH independent. As expected, endocytosis of transferrin, a marker of CME, was inhibited by CPZ and dynasore (Extended Data Fig. 8), whereas lactoceramide, a marker of caveolin-mediated endocytosis, was blocked by dynasore (Extended Data Fig. 9). The endocytosis inhibitors did not affect the localization of specific markers of mitochondria (E1 alpha pyruvate dehydrogenase), lysosomes (LAMP1), Golgi (human Golgi-resident enzyme N-acetylglucosaminyltransferase 2), peroxisomes (peroxisomal C-terminal targeting sequence), EE (RAB5A) or LE (RAB7A), except for dynasore, which, as expected, affected the accumulation of RAB5A in the EE compartment (Extended Data Fig. 10). Taken together, these data show that exo-HAV enters the cell by CME and that infectivity is mediated by RNA cargo delivery into the cytoplasm (Fig. 2) via a fusion mechanism (Fig. 3) at the LE or a later compartment, evading degradation of the exo-HAV membrane and the capsid-free HAV RNA in the lumen by lysosomal lipases and nucleases.

The role of exo-HAV in the life cycle of HAV is unclear. HAV is not unique in producing infectious exosomes, which are also produced by enveloped viruses such as hepatitis C virus (HCV) and non-enveloped picornaviruses such as poliovirus and coxsackievirus, which have very different pathogenic processes. Exosomes from virus-infected cells can induce innate immune responses in non-permissive bystander cells that express HAVCR1, such as the activation of plasmacytoid dendritic cells by exosomes from cells infected with HCV17 or Epstein–Barr virus containing a cargo of immunostimulatory small RNAs18. Although plasmacytoid dendritic cells preferentially sense exo-HAV and produce interferon-α (INF-α)19, the role of type I IFN in HAV infection is unclear since patients with different degrees of disease severity...
show significant differences in IFN-level responses, and infection of chimp s with HAV results in a limited IFN response. Clearly, exo-HAV has no role in the fecal–oral route of transmission of HAV because the excreted virus is depleted from lipid membranes in the digestive tract. Therefore, further research is needed to understand the role of exo-HAV in the pathogenesis of HAV.

Here, we used CRISPR–Cas9 gene KO technology and exosomes from HAV-infected cells to study RNA cargo delivery from the exosome lumen into the cytoplasm. This mechanism mediated by cellular receptors is poorly understood, paramount to our understanding of the biology of exosomes and is shared by viruses to infect cells. We determined that two lipid receptors, HAVCR1 and NPC1, are required for the delivery of RNA from the lumen of exosomes into the cytoplasm by a fusion mechanism that is independent of viral envelope GPs. EBOV and other filoviruses also use the HAVCR1–NPC1 pathway for the delivery of genetic material into the cytoplasm, but in the context of the viral GP interaction with NPC1, an endosomal receptor that is thought to trigger EBOV GP conformational changes responsible for the fusion of the viral and LE membranes. However, the endosomal uncoating mechanism of EBOV mediated by the interaction of GP with NPC1 is far from clear. Our results indicated that fusion of the delimiting membranes of the exosome and LE that leads to cargo delivery of RNA and exo-HAV infection is mediated by HAVCR1 and NPC1 in the absence of a viral GP. This finding suggests that the EBOV GP–NPC1 interaction is not necessary for fusion but may be required for other functions, such as stabilizing or enlarging pores for the transfer of a large ribonucleoprotein from the lumen of the viral particle into the cytoplasm and leading to productive infection. Indeed, this envelope-glycoprotein-independent exosome mimic pathway of cargo delivery using multiple lipid receptors may represent a common mechanism for cell entry of other viruses that are difficult to protect against, such as HIV and HCV, which uses NPC1-like 1 cholesterol transporter and HAVCR1 as cell entry factors.

We recently showed that the KO of HAVCR1 in AGMK cells prevents vPhAV and exo-HAV infection, and that transfection of HAVCR1 or its mouse orthologue into AGMK HAVCR1 KO cells restores their infectivity. Our positive data confirmed that HAVCR1 is a functional HAV receptor in AGMK cells. This differs from the findings of Lemon and Maury and colleagues, who showed that truncation of HAVCR1 in HuH7 and Vero E6 cells does not interfere with HAV entry. However, truncation of viral receptors is often not sufficient to abrogate their function, and both HuH7 and Vero E6 are known to express additional as yet unidentified receptors for vPhAV. Here, we showed that HuH-7 cells require HAVCR1 for exo-HAV but not vPhAV infection, which provides further evidence to indicate that the HAVCR1–NPC1 pathway is used by exosomes to deliver their luminal cargo into the cytoplasm of the cell.

Our data support an exosome cargo delivery model mediated by two lipid receptors: HAVCR1 and NPC1 (Fig. 4d). It is possible that this fusion mechanism acts independently of or in conjunction with the SNARE-mediated and viral GP-mediated mechanisms used by cells and viruses for membrane fusion and cargo delivery. Further research is required to identify other factors involved in the HAVCR1–NPC1 pathway to allow a complete understanding of the exosome cargo delivery mechanism and define therapeutic targets to prevent pathogenesis.

Methods

**Cells and viruses.** HuH7 cells (catalogue number (cat. no.) CBR0403, Japanese Collection of Research Bioresources) selected for the stable growth of wild-type HAV were maintained in DMEM (Gibco). The clone GL37 of AGMK cells was passaged 100 times in cell culture (HAV PI) and grown in AGMK cells. A recombinant cell-culture-adapted HAV containing a Bsd selectable marker, 3H Av/Bsd, was derived from infectious cDNA as described above and grown in AGMK cells in the presence of 5 µg ml⁻¹ Bsd (ThermoFisher Scientific). The recombinant wild-type HAV containing a Bsd-selectable marker, 3H Av/Bsd, was also derived from infectious cDNA as described above and grown in HuH7 cells in the presence of 5 µg ml⁻¹ Bsd. Infectious viral titres were determined by the haemagglutination inhibition assay (AHA).

VSV pseudotypes containing the green fluorescence protein (GFP) gene and the VSV-G envelope (VSV–GFP) or the Zaire Ebola virus glycoprotein (rVSV–EBOVgp–GFP) were produced in Vero E6 cells. Viral titres were determined using the online ID50 program (https://www.ncbi.nlm.nih.gov/ CRBResearch/Spouge/html/ncbi/html/id50/id50.cgl) developed by J. L. Spouge.

**Flow cytometry analysis.** Mouse anti-human HAVCR1 (CD365) 1D12 monoclonal antibody (mAb) (clone 1D12, BioLegend) reacts against an IgV-like domain epitope conserved between human and monkeys. HAVCR1-1 mAb was raised against a HAVCR1-FC fusion protein, reacted against the IgV domain of HAVCR1, protected cells against HAV infection and blocked the binding of apoptotic cells. Biotin-labelled goat anti-human HAVCR1 1750 antibody (R&D Systems) reacts against the IgV and mucin-like domains of HAVCR1. Parental cells and vector- or HAVCR1-transfected cells were stained with 1D12, HAVCR1-1 or mouse IgG1 isotype control (clone 15H6, Southern Biotech) mAbs as primary antibodies and PE-labelled mouse polyclonal IgG (Southern Biotech) as secondary antibody. Vector- , HAVCR1- or HAVCR1ΔGp/VAX5-transfected cells were stained with biotin-conjugated 1750 antibody as a primary antibody and Alexa Fluor 488-labelled streptavidin (ThermoFisher Scientific). Alternatively, cells were stained with the PE-labelled anti-HAVCR1 mAb 1D12 (clone 1D12, ThermoFisher Scientific).

To determine tetraspanin expression at the cell surface of HuH7 cells, monolayers of HuH7 cells were dislodged with 0.5 mM EDTA, washed with 5% FBS–PBS and stained with murine PE-labelled anti-human CD9 (clone M-L13, Southern Biotech), CD63 (clone H5C6, BD Biosciences) or CD81 (clone JS-81, BD Biosciences) mAbs as recommended by the manufacturer. PE-labelled anti-human CD3 (clone UC171, BD Biosciences) was used as the isotype control following the manufacturer’s recommendation.

**Lapatinib-assisted flow cytometry analysis of exosomes was performed as previously described, but with minor modifications. Aliquots (one-tenth of each fraction, 20 µl) of iodixanol density gradient fractions were adsorbed to 2 µl of 4 µM aldehyde/sulfate latex beads, 4% w/v (ThermoFisher Scientific), and stained with PE-labelled anti–CD1d, -CD9, -CD63 or -CD81 mAbs. Median fluorescence intensity (MFI) was determined for each fraction. Antibody-stained cells and exosome-bound latex beads were analysed by flow cytometry in a Guava EasyCyte instrument using CytoSoft v.5.3 software (EMD Millipore). Caspase-3 staining was analysed by flow cytometry in a FACScanto II using FACSDiva v.8.0.1 software (BD Biosciences). Data analysis was performed using FlowJo software (Becton, Dickinson and Company) to 10^6 apoptotic cells and v.8.5 for caspase-3 stains.

**Liposome fusion experiments.** For fusion studies of HEK-293 cells (Fig. 1b) and fusion kinetics of HuH7 cells (Fig. 3c), liposomes were prepared using the lipid extrusion method and an Avanti Mini-Extruder kit (Avanti Polar Lipids) with a 3:1 (w/w) mixture of PE (Avanti Polar Lipids) and Chol (Avanti Polar Lipids) at 1:1 molar ratio of 12:12:1:2 as recommended by the manufacturers. Quality control of r18-labeled PS:PC:Chol liposomes was performed by in vitro fluorescence
dequenching in liposomes treated with 1% Triton X-100 for 15 min at room temperature (t.t.) in a 96-well plate. An approximate fivefold increase in fluorescence was determined in a Synergy HT fluorescence plate reader (BioTek Instruments) using 530-nm excitation/590-nm emission filters and Gen5 software, which indicated that the R8B bound to the liposomes was adequately quenched.

Cells grown on glass coverslips were incubated with R8B-labeled liposomes for 10 min at t.t., washed, incubated at 37 °C under 5% CO2 for different times (0, 15, 30, 45 or 60 min) and fixed with 3% paraformaldehyde (PFA). Cells were mounted on slides using ProLong Gold antifade reagent with DAPI (ThermoFisher Scientific) as a nuclear counterstain. Slides were analysed using a LSM 710 confocal microscope and ZEN 2011 software (Carl Zeiss). Micrographs were taken with a ×40 oil-immersion objective.

To assess phospholipid content, temperature, pH dependency, endosome inhibitors or HAVCR1 and NPC1 requirement for liposome fusion, Huh7 parental or KO cells were grown in 12-well glass chamber slides, and treated with commercially available R8B-labelled liposomes (Encapsula NanoSciences). For the phospholipid-content dependency studies, 50 µM of R-18 liposomes containing PS, PC, and Chol (PS:PC:Chol=1:1:1) were added to the cells, incubated for 30 min at 37 °C, washed, and incubated for 30 min at 37 °C, 15 °C or 4 °C. For the pH dependency studies, cells were treated for 6 h at 37 °C with 2 µM monomannos (ThermoFisher Scientific) or overnight at 37 °C with 30 µM mannosylate (ThermoFisher Scientific) or overnight at 37 °C with 30 µM monomannos +50 µM choline chloride, then treated with 50 µM PS:PC:Chol=1:1:1 for 15 °C for 10 min, and washed and incubated for 30 min at 37 °C. After incubation, cells were washed three times with PBS. To determine whether liposome fusion occurs at the cell surface or intracellularly and to quantitate fusion of liposomes in Huh7, HAVCR1 or NPC1 KO cells, cells were treated with 50 µM PS:PC:Chol-R18 for 10 min at 15 °C, washed, incubated or not for 30 min at 37 °C, washed, and stained with CellMask (ThermoFisher Scientific) for 15 min at 37 °C and extensively washed. For these liposome fusion studies, cell nuclei were counterstained with Deep Red Anthraquinone 5 (DRAQ5, Thermofisher Scientific) or DAPI, and cells were fixed with 4% PFA, coverslips were mounted with ProLong Gold antifade reagent (Life Technologies) and analysed using a LSM 700 confocal microscope and ZEN 2011 software (Carl Zeiss). Micrographs were taken with a ×40 oil objective. R8B fluorescence due to liposome fusion was quantitated using ImageJ v 1.8.0 (Rasband, WS., ImageJ, US National Institutes of Health; https://imagej.nih.gov/ij/, 1997–2018).

The concentration of 2 µM monomannos, 30 µM chloroquine diphosphate and 5 mM ammonium chloride in Huh7 cells was determined by flow cytometry using a caspase-3 apoptosis assay. Briefly, cells were trypsinized after treatment with the compounds or heat treated for 7 min at 55 °C (apoptosis/dead induction control), stained with 1 µl of the cell-permeable caspase-3 inhibitor DEVD-FMK conjugated to sulfo-rhodamine (Red–DEVD-FMK) that binds irreversibly to activated caspase-3 (CaspGLOW Red Active Caspase-3 Staining kit, BioVision), incubated at 37 °C for 45 min, washed three times and analysed using a FACS Canto II (BD Biosciences) instrument and FlowJo v.8.5 (Becton, Dickinson and Company).

HAV-specific RT–qPCR assay. RNA from gradient fractions was extracted using a High Pure Viral RNA Test Kit (Roche Diagnostics). Alternatively, samples from gradient fractions were diluted 1:5 in RNase-free water and heated at 95 °C for 5 min before RT–qPCR analysis. Total RNA from cell lysates was isolated using a RNasy easy kit (Qiagen). HAV RNA content was determined by RT–qPCR using HAV-specific primers and FAM/MGB probe® and a RNA UltraSense one-step quantitative RT–PCR kit (ThermoFisher Scientific). Intracellular HAV RNA was normalized to total extracted RNA. Alternatively, samples were amplified with a known amount of poliovirus, which was used to correct HAV values according to the extraction efficiency. The RT–qPCR analysis was performed in a QuantStudio 6 Flex Real-Time PCR system (ThermoFisher Scientific).

Purification and characterization of exosomes and viral particles from HAV-infected cells. Exosomes and viral particles from HAV-infected cells were extensively characterized1,2, and we used the same cells, virus strains and purification procedures to produce exosomes for this study3,4,5,6. These purified exosomes had the same density, content of AchE (an exosome marker7,8), content of HAV RNA and infectivity characteristics of the published exosomes. Exosomes and viral particles from HAV-infected cell culture supernatants were produced as previously described3,4. Exosomes and HAV particles were purified from HAV/BS-infected AGMK cells (exo-HAV/Bsd and vPHV/Bsd, respectively), HAV8/bd-infected Huh7 cells (exo-HAV and vPHV, respectively) or HAV-PI-infected AGMK cells (exo-HAV PI and vPHV PI, respectively). Briefly, cell supernatants collected at 8 days post-infection (p.i.) were clarified by centrifugation at 1,000 × g and twice at 10,000 × g and ultracentrifuged at 100,000 × g for 4 h at 4 °C. Pellets containing exosomes and viral particles were resuspended in PBS, loaded onto 8–40% iodixanol (OptiPrep Density Gradient Medium, Sigma-Aldrich) step gradients and separated by isopyric ultracentrifugation at 141,000 × g for 18 h at 4 °C. Approximately 20–24 fractions were collected from each gradient and analysed by HAV RT–qPCR and for AchE content using a colorimetric Acetylcholinesterase Assay kit (Abcam). The density of each gradient fraction was determined using a refractometer (Portlab Lab, Mettler Toledo), and the peaks of exosomes (density of 1.04–1.06 g cm−3) and AchE, and HAV particles (density of 1.27 g cm−3), which contained HAV RNA but not AchE, were collected and stored at 4 °C for further experimentation.

The presence of PS at the surface of exosomes purified from HAV-infected cells was analysed using the annexin V (ANX5)-based assay kit EasySep Dead Cell Removal Kit (Stemcell Technologies) and an immunofluorescence separation method based on three components: biotinylated ANX5 that binds PS, a cocktail of anti-biotin mAbs that bind to the biotinylated ANX5 and magnetic beads that bind the mAbs. Purified exo-HAV/Bsd (0.020 ml per sample) in 0.1 ml of ANX5 binding buffer (BioLegend) was incubated with or without biotinylated ANX5 for 5 min at r.t. in the presence or not of the anti-biotin mAbs. Magnetic beads were added to all samples and washed for 5 min at r.t., then beads were separated using a magnet and washed twice with ANX5 buffer. RNA from exo-HAV/Bsd bound to the magnetic beads was purified using a RNeasy Mini kit (Qiagen) as recommended by the manufacturer, and quantitated by HAV RT–qPCR.

The tetraspanin enrichment at the surface of exosomes was determined using labelled flow cytometry staining with anti-CD9, -CD63 and -CD81 mAbs as described above (see “Flow cytometry analysis” section).

Western blot analysis of iodoxanol density gradient fractions was performed using one-fifth to one-tenth of each fraction resolved by denaturing SDS–PAGE in Bolt 4–12% Bis-Tris minigels, transferred to polyvinylidenedifluoride membranes using a iBlot Dry Blotting System (Life Technologies) or 5% or 20% SDS polyacrylamide gels (BioLegend), and blocked for 30 min with 5% or 20% BSA in TBS-T. Detection was performed using a RNeasy Mini kit, and HAV RNA was quantitated by HAV RT–qPCR.

The Bsd-resistance CFU assay was performed as previously described11, but with minor modifications. For AGMK cells, cells were infected at 48 h post-transfection (p.t.), virus was absorbed for 12 h and incubated at 37 °C for 72–96 h before splitting. For Huh7 parental cells, Huh7 HAVCR1 KO or Huh7 NPC1 KO cells in 12-well plates were treated with purified exo-HAV or vPHV for 2h, washed, incubated at 37 °C under 5% CO2 for 24 h, trypsinized, seeded at different densities (2 × 104, 1 × 105 or 5 × 105 cells per well) in 6-well plates, and grown in cell culture media containing 5 µg ml−1 Bsd for 10–12 days. Cells were fixed with 80% methanol, stained with crystal violet, and CFUs from duplicate wells were manually counted and imaged using a flatbed scanner.

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respectively (see ref. 59 and references therein). For transferrin uptake assays, fluorescence plate reader using Gen5 software. With VSV pseudotypes”) and GFP fluorescence quantitated in a Synergy HT Bioscience) as recommended by the manufacturer.

Inhibition of cell entry. Treatments of exo-HAV and vpHAV with 200 g ml–1 of tetramethylrhodamine dichloroacetate (CMFDA; ThermoFisher Scientific). Subconfluent monolayers of Huh7 parental, HAVCR1 KO or NPC1 KO cells in 12-well plates were incubated with 104 apoptotic cells for 30 min at r.t., non-transfected cells were washed and the bound apoptotic cells were quantitated in a Synergy HT fluorescence plate reader using 485-nm/528-nm excitation/emission filters. Monolayers were also analysed using an Axiovert 200 fluorescence microscope (Carl Zeiss) and micrographs were taken with a x20 objective using AxioVision software (Carl Zeiss).

Infection with VSV pseudotypes. To assess the susceptibility to EBOV infection, Huh7 and Huh7 NPC1 KO cells in 6-well plates were infected with rVSV-EBOVgp–GFP at a m.o.i. of 5 medium tissue culture infectious dose (TCID₅₀) per cell and incubated at 37°C under 5% CO₂. GFP fluorescence was determined at different times p.i. in a Synergy HT fluorescence plate reader and expressed as MFI averages.

For flooding studies, Huh7 cells seeded in 24-well plates for 48 h were treated with different concentrations of cell entry inhibitors for 1 h at 37°C. Either exo-HAV or pHV was added and incubated for 2 h at 37°C. Monolayers were extensively washed, fed with DMEM supplemented with 10% FBS and the corresponding inhibitor, and incubated at 37°C for 3 days under 5% CO₂. Total RNA was extracted from cells and HAV RNA was quantified by RT-qPCR.

To confirm the activity of the compounds, Huh7 cells were treated with the inhibitors as described above, infected with rVSV-EBOVgp–GFP (see “Infection with VSV pseudotypes”) and GFP fluorescence quantitated in a Synergy HT fluorescence plate reader using Gen5 software.

The specificity of the inhibition was tested by endocytosis of human transferrin and lactosylceramide as markers of CME and caveolin-mediated endocytosis, respectively (see ref. 2 and references therein). For transferrin uptake assays, Huh7 cells grown in 8-well glass chamber slides were treated with pre-warmed transferrin-staining medium (25 mM HEPES pH 7.4, 0.5% BSA) for 1 h at 37°C before the addition of 5g ml–1 CPZ, 80 µM dynasore, 10 µM EGA, 40 µM EIPA, 10 µM LY29402 or DMSO (control). After incubation for 15 min at 37°C in the presence of the inhibitors, 25g ml–1 of tetracycline/trimethoprim conjugate-transferrin (Tritec-Transferrin) (ThermoFisher Scientific) in transferrin-staining medium was added to the cells and incubated for 15 min at 37°C. Cells were washed with cold 0.2M sodium acetate pH 4.5 and twice with cold PBS, stained with DRAQ5 as a nuclear counterstain, fixed with 4% PFA and mounted with ProLong Gold antifade reagent. For lactosylceramide uptake assays, Huh7 cells grown in 8-well glass chamber slides were incubated with BODIPY FL C5-lactosylceramide complexed to BSA (LAC-cer) (ThermoFisher Scientific) for 30 min at 4°C, washed with cold PBS, treated with 5g ml–1 CPZ, 80 µM dynasore, 10 µM EGA, 40 µM EIPA, 10 µM LY29402 or DMSO (as control) for 45 min at 37°C. After incubation, cells were washed with PBS, stained with DRAQ5 as a nuclear counterstain, fixed with 4% PFA and mounted with ProLong Gold antifade reagent. Micrographs were taken with an LSM 780 confocal microscope using a ×40 oil objective.

Detergent and RNase treatment of exosomes. Control vphHAV and exo-HAV were treated with 1% sarkosyl (Sigma-Aldrich) for 1 h at r.t. followed by treatment with 50g ml–1 of RNase A (Sigma-Aldrich) for 30 min at 37°C. Samples were separated by ultracentrifugation through a 40% sucrose (Sigma-Aldrich) cushion at 120,000 r.p.m. for 15 min at 4°C in a Sorvall MX 120 Plus Micro-Ultracentrifuge (ThermoFisher Scientific). The cell surface of transfected cells was determined by flow cytometry at 48h p.i., staining cells with PE-labelled anti-HAVCR1 IgV mAb 1D12. Transfectants were also stained with unlabelled anti-HAVCR1 1D12 and primary antibody and PE-labelled anti-mouse antibody as secondary antibody or biotin-conjugated 1750 antibody as primary antibody followed by Alexa Fluor 488-labelled streptavidin.

MB inactivation. Incubation of exo-HAV and pHV with 2µM MB (Sigma-Aldrich) occurred for 20 min at r.t. in the dark, and photoinactivated or not (flash negative controls) for 20 min with fluorescent white light at r.t. as previously described32. Remaining HAV infectivity in Huh7 cells was determined by ARTA49.

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DPM—RT-qPCR assay. Treatment of exo-HAV and pHV with 200µM PMA (Biotium) occurred for 15 min at r.t. in the dark with constant agitation, and exposed or not (flash negative controls) to a 500-W light for 15 min on ice. Samples treated at 85°C for 5 min to expose HAV RNA before incubation with PMA were used as positive inactivation controls.

Inhibition of cell entry. The cell entry inhibitors CP2 hydrochloride (Sigma-Aldrich), dynasore hydrazide (Sigma-Aldrich), LY29402 (Calbiochem), EIPA (Sigma-Aldrich) and EGTA (Calbiochem) were prepared as suggested by the manufacturers. As negative controls, cells were treated with dimethylsulfoxide (DMSO; Sigma-Aldrich) at the same concentration used for the compounds. Huh7 cells seeded in 24-well plates for 48 h were pretreated with different concentrations of cell entry inhibitors for 1 h at 37°C. Either exo-HAV or pHV was added and incubated for 2 h at 37°C. Monolayers were extensively washed, fed with DMEM supplemented with 10% FBS and the corresponding inhibitor, and incubated at 37°C for 3 days under 5% CO₂. Total RNA was extracted from cells and HAV RNA was quantified by RT-qPCR.

Cytotoxicity of the compounds in Huh7 cells was determined using a Dual-fluorescence Acridine Orange/Propidium iodide assay (NeXelom Bioscience) as recommended by the manufacturer.

To confirm the activity of the compounds, Huh7 cells were treated with the inhibitors as described above, infected with rVSV-EBOVgp–GFP (see “Infection with VSV pseudotypes”) and GFP fluorescence quantitated in a Synergy HT fluorescence plate reader using Gen5 software. The specificity of the inhibition was tested by endocytosis of human transferrin and lactosylceramide as markers of CME and caveolin-mediated endocytosis, respectively (see ref. 2 and references therein). For transferrin uptake assays, Huh7 cells grown in 8-well glass chamber slides were treated with pre-warmed transferrin-staining medium (25mM HEPES pH 7.4, 0.5% BSA) for 1 h at 37°C before the addition of 5g ml–1 CPZ, 80µM dynasore, 10µM EGA, 40µM EIPA, 10µM LY29402 or DMSO (as control). After incubation for 15 min at 37°C in the presence of the inhibitors, 25g ml–1 of tetracycline/trimethoprim conjugate-transferrin (Tritec-Transferrin) (ThermoFisher Scientific) in transferrin-staining medium was added to the cells and incubated for 15 min at 37°C. Cells were washed with cold 0.2M sodium acetate pH 4.5 and twice with cold PBS, stained with DRAQ5 as a nuclear counterstain, fixed with 4% PFA and mounted with ProLong Gold antifade reagent. For lactosylceramide uptake assays, Huh7 cells grown in 8-well glass chamber slides were incubated with BODIPY FL C5-lactosylceramide complexed to BSA (LAC-cer) (ThermoFisher Scientific) for 30 min at 4°C, washed with cold PBS, treated with 5g ml–1 CPZ, 80µM dynasore, 10µM EGA, 40µM EIPA, 10µM LY29402 or DMSO (as control) for 45 min at 37°C. After incubation, cells were washed with PBS, stained with DRAQ5 as a nuclear counterstain, fixed with 4% PFA and mounted with ProLong Gold antifade reagent. Micrographs were taken with an LSM 780 confocal microscope using a ×40 oil objective.

To confirm the activity of the compounds, Huh7 cells were treated with the inhibitors as described above, infected with rVSV-EBOVgp–GFP at a m.o.i. of 5 medium tissue culture infectious dose (TCID₅₀) per cell and incubated at 37°C under 5% CO₂. GFP fluorescence was determined at different times p.i. in a Synergy HT fluorescence plate reader and expressed as MFI averages.

To confirm the activity of the compounds, Huh7 cells were treated with the inhibitors as described above, infected with rVSV-EBOVgp–GFP at a m.o.i. of 5 medium tissue culture infectious dose (TCID₅₀) per cell and incubated at 37°C under 5% CO₂. GFP fluorescence was determined at different times p.i. in a Synergy HT fluorescence plate reader and expressed as MFI averages.
incubated at 37 °C for 24h. Supernatant samples of the infected cells were taken at 24h p.i. and titrated by an endpoint dilution assay in 96-well plates containing subconfluent monolayers of HuH7 cells using tenfold dilutions in triplicate wells. After 72 h of incubation at 37 °C, 96-well plates were transfected using an Axiom 200 fluorescence microscope to determine green fluorescence and cytopathic effect (CPE). Viral titres were determined using the online ID50 program (https://www.ncbi.nlm.nih.gov/CBResearch/Spouge/html.ncbi/html/d0/d0.cgi).

Effect of NPC1 mutations on exo-HAV infection. To analyse the effect of NPC1 mutations on exo-HAV infection, HuH7 NPC1 KO cells in 12-well plates were transfected with pEAK12-Hyg NPC1-mKG(N), plasmids containing single or double mutations in NPC1 or vector. At 48 h.p.i., cells were extensively washed, infected with exo-HAV at a m.o.i. 0.5-1 for 24 h, split 1:4 into 6-well plates and incubated for 96h at 37 °C. Total RNA was extracted from cells using a RNaseasy Mini kit and HAV infection quantitated by HAV RT–qPCR.

Statistical analysis. Statistical methods were not used to predetermine sample sizes and experiments were not blinded to allocation or accessing outcome. All experiments were conducted at least three times, and the mean values and the standard deviation (s.d.) were calculated using Prism 8 (GraphPad Software) or Excel (Microsoft) software. Statistical significance of log-transformed normalized data was calculated using one-way analysis of variance (ANOVA) with Dunnett’s post-test. For nonparametric data, statistical significance was assessed by two-sided Mann–Whitney test or Kruskal–Wallis test, a nonparametric equivalent of one-way ANOVA. Box and whiskers plots were generated using the Tukey method to graph whiskers and outliers. Statistical analyses were performed using Prism 8 software.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The authors declare that the data supporting the findings of this study are available from the corresponding author upon reasonable request. Numerical and statistical source data that underlie the graphs in figures, extended data and supplemental data are provided with the paper.

Received: 27 May 2019; Accepted: 12 May 2020; Published online: 15 June 2020

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Acknowledgements
This work was supported by funding from the US Food and Drug Administration Intramural Program to G.K. and a Medical Countermeasures Initiative to G.K. This project was supported in part by appointments to the Research Fellowship Program at the Office of Blood Research and Review, Center for Biologics Evaluation and Research, US Food and Drug Administration, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the US Department of Energy and the FDA (to M.I.C., A.A. and H.L.).

Author contributions
M.I.C. and G.K. were responsible for the overall design of the study. M.I.C. carried out most of the experiments, and A.A. and H.L. performed the confocal microscopy studies. G.K. performed the studies with NPC1 mutants, block of infectivity by liposomes and characterization of exosomes by western blot analysis and flow cytometry. M.I.C., A.A. and G.K. analysed the data. G.K. wrote the manuscript and M.I.C helped with the editing. All authors reviewed and commented on the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41564-020-0740-y;
Supplementary information is available for this paper at https://doi.org/10.1038/s41564-020-0740-y;
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Extended Data Fig. 1 | Fusion of R18-labeled liposomes is temperature dependent and requires phosphatidylserine and cholesterol. a, Huh7 cells were treated with PS:PC:Chl-R18 liposomes for 30 min at 37°C, 15°C, or 4°C to test for liposome fusion (red). Nuclei were stained with DAPI (blue). Cells were analyzed in a LSM 700 confocal microscope. Micrographs were taken using a 40x oil objective. 
b, Quantitative analysis of the fusion of R18-labeled liposomes in endosomes from (a). The R18 fluorescence intensity (red) of 20 cells from (a) was measured using ImagJ software and the experiment was repeated 3 times (n = 60). 
c, Huh7 cells were treated with PS:PC:Chl-R18, PS:PC-R18, PC:Chl-R18, or PC-R18 during 30 min at 37°C. Nuclei were stained with DAPI (blue). Cells were analyzed in a LSM 700 confocal microscope. Micrographs were taken with a 40x oil objective. 
d, Quantitative analysis of the fusion of R18-labeled liposomes in endosomes from (c). R18 fluorescence intensity (red) of 20 cells from (c) was measured using ImagJ software, and the experiment was repeated 3 times (n = 60). Scale bars in (a) and (c) represent 50 μm. In (b) and (d), box and whiskers plots were done using the Tukey method. Box limits are upper and lower quartiles, center lines are the medians, whiskers are 1.5x interquartile range, and points represent outliers. P values were determined by two-sided Mann-Whitney test.
Extended Data Fig. 2 | Intracellular fusion of R18-labeled liposomes is pH-dependent. a, Liposome fusion (red) in Huh7 cells treated with 2 μM Monensin, 30 μM Chloroquine diphosphate, or 5 mM Amonium chloride (NH₄Cl) prior to the addition of PS:PC:Chl-R18 liposomes. Nuclei were stained with DAPI (blue). Cells were analyzed in a LSM 700 confocal microscope. Micrographs were taken using a 40x oil objective. Scale bars represent 50 μm. b, Quantitative analysis of the fusion of R18-labeled liposomes in endosomes from (a). The R18 fluorescence intensity (red) of 20 cells from (a) was measured using ImageJ software and the experiment was repeated 3 times (n=60). Box and whiskers plot was done using the Tukey method. Box limits, upper and lower quartiles; Center line, median; whiskers, 1.5x interquartile range; points, outliers; P values between untreated and treated cells were determined by two-sided Mann-Whitney test. c, Lack of cytotoxicity of the compounds was confirmed by flow cytometry using a caspase-3 apoptosis assay based on the irreversible binding of cell-permeable inhibitor DEVD-fmk conjugated to sulfo-rhodamine (Red-DEVD-fmk) to activated caspase-3. Stained cells were analyzed with a FACSCanto II (BD Biosciences) using FlowJo v8.5 software. Apoptotic/dead Huh7 control cells were prepared by heat treatment at 55 °C for 7 min. d, Liposome fusion with intracellular membranes. Huh7 cells were treated with PS:PC:Chl-R18 liposomes (R18 liposome fusion events, red) for 15 min at 37 °C, the plasma membrane was stained with CellMask (green) for 15 min at 37 °C, and nuclei were stained with DRAQ5 (blue). Cells were analyzed in a LSM 700 confocal microscope. Micrographs were taken with 40x oil objective. Scale Bars represent 25 μm. Liposome fusion events detected by R18 fluorescence (red) that did not co-localize with plasma membrane stained with Cell Mask (green). Results are representative of 3 independent experiments.
Extended Data Fig. 3 | Characterization of exosomes purified from HAV-infected cells. 

**a**, Presence of PS at the surface of exosomes from HAV-infected cells. Purified exo-HAV/Bsd treated with (+) or without (-) biotinylated ANX5 (ANX5-Biotin) and anti-biotin mAbs was bound to magnetic beads, washed extensively, and extracted HAV RNA was quantitated by RT-qPCR. Data are mean ± sem, n=3. P values between complete and incomplete immunomagnetic sandwiches were analyzed by one-way ANOVA with Dunnett’s post-test.

**b**, Purification by isopycnic ultracentrifugation of supernatants of HAV/Bsd-infected Huh7 cells. Gradient collected in 25 fractions from the top. HAV RNA in fractions 5–24 was quantified by RT-qPCR showing peaks of exo-HAV (fractions 11–13, 1.08–1.10 g cm-3) and vpHAV (fraction 21–23, 1.23–1.33 g cm-3). Data are mean ± sd of RT-qPCR duplicates.

**c**, Enrichment of exosomal markers HSP70, FLOT-1, and TSG101 but not Golgi marker GOLGA1 in exo-HAV assessed by Western blot analysis of gradient fractions 5–25 from (**b**). Fractions aligned with (**b**). Cell extracts from uninfected Huh7 cells were used as controls. Migration of molecular weight markers is shown in kDa.

**d**, Flow cytometry analysis of cell surface expression of CD9, CD63, and CD81 on Huh7 cells using PE-labeled anti-human CD9, CD63, or CD81 mAbs compared to a PE-labeled isotype control. Gating strategy in left panel.

**e**, Flow cytometry analysis of gradient fractions 2–24 from (**b**) absorbed to latex-beads and stained with mAbs as in (**d**). Gating strategy in left panel.

**f**, Content of CD63 and CD81 on exo-HAV (fraction 11 from (**b**)) compared to control vpHAV (fraction 22 from (**b**)) by flow cytometry as described in (**e**). Gating strategy in left panel.

**g**, Sizing of exo-HAV (fractions 11–13 from (**b**)) by DLS analysis in a Zetasizer nano ZS90 instrument performed in 3 runs (red, blue, and green lines) of 10 measurements each. Data are representative of 3 independent experiments.
Extended Data Fig. 4 | HAVCR1 is an exo-HAV cellular receptor. **a**, Specific infectivity of purified exo-HAV and vpHAV from Fig. 1c in Huh7 cells was determined as the ratio between HAV RNA genome equivalents (GE) assessed by RT-qPCR and infectious particles assessed by ARTA. Data are mean ± sem, n=5 from 5 different titrations. **b**, Expression of HAVCR1 but not HAVCR1 N94A restored exo-HAV cell entry in Huh7 HAVCR1 KO cells. HAVCR1, HAVCR1 N94A, or vector transfectants were infected with exo-HAV or vpHAV from Fig. 1c, and tested for Bsd-resistant CFU. Data are mean ± sem, n=4 from two independent experiments with biological duplicates. **c**, Flow cytometry analysis of the cell surface expression of HAVCR1 (red dots) or HAVCR1DIgV/ANX5 (blue dots) compared to vector-transfected cells (grey dots) on Huh7 HAVCR1 KO cell transfectants stained with pE-labeled anti-HAVCR1 mucin mAb1750. Gates contain cells expressing the HAVCR1 mucin 1750 epitope. Data representative of 4 independent experiments. **d**, Huh7 HAVCR1 KO cell transfectants expressing HAVCR1 or HAVCR1DIgV/ANX5 bind apoptotic cells. Monolayers of vector, HAVCR1, or HAVCR1DIgV/ANX5 cell transfectants or control Huh7 parental cells were incubated with CMFDA-labeled apoptotic Jurkat cells and washed extensively. Phase contrast (DIC) and green fluorescence (CMFDA) micrographs were taken with an Axiovert 200 fluorescence microscope using a 20x objective. Scale bar represents 50 mm. Data representative of 3 independent experiments. **e**, Expression of an annexin V fusion protein at the cell surface restores infectivity of exo-HAV. Huh7 HAVCR1 KO cell transfectants from (**c**) were infected with purified exo-HAV from Fig. 1c using a m.o.i of 0.1-0.5 for 48 h at 37°C. Total RNA was extracted from the cells and HAV RNA was quantitated by HAV RT-qPCR. Data are mean ± sem, n=4 from 2 independent experiments with 2 biological replicates. P values were determined by two sided Mann-Whitney test.
Extended Data Fig. 5 | Knock out of NPC1 in Huh7 cells blocks EBOV cell entry and endosomal liposome fusion. a, Growth of rVSV-EBOVgp-GFP in Huh7 parental and NPC1 KO cells. Huh7 parental and NPC1 KO cells were infected with rVSV-EBOVgp-GFP for 24 h. At different times p.i., GFP fluorescence was assessed using a fluorescence plate reader. Data are mean ± sem, n=4 from 4 independent experiments. P values between Huh7 parental and NPC1 KO cells were determined by two-sided Mann-Whitney test. b, Quantitative analysis of the fusion of R18-labeled liposomes in endosomes. Huh7 parental, HAVCR1 KO and NPC1 KO cells were treated with PS:PC:Chl-R18 liposomes 10 min at 15 °C and incubated or not for 30 min at 37 °C to test for liposome fusion (red). Nuclei were stained with DAPI (blue). Cells were analyzed using a LSM 700 confocal microscope (Carl Zeiss), and micrographs were taken using a 40x oil objective. Scale bar represents 50 mm. c, Quantitative analysis of the fusion of R18-labeled liposomes in endosomes from (b). The R18 fluorescence intensity (red) of 25 cells was measured using ImageJ software, and the experiment was repeated 3 times (n=75). Box and whiskers plots were done using the Tukey method. Box limits, upper and lower quartiles; Center line, median; whiskers, 1.5x interquartile range; points, outliers; P values between mean fluorescence intensity (MFI) of Huh7 HAVCR1 KO or Huh7 NPC1 KO cells compared to Huh7 parental cells were determined by two-sided Mann-Whitney test.
Extended Data Fig. 6 | Interaction of NPC1 with HAVCR1 in Huh7 NPC1 KO cell transfectants. a, Schematic representation of bimolecular fluorescence complementation (BiFC) assay based on the complementation of the monomeric Kusabira green (mKG) protein (168 amino acid (aa) N-terminus fragment (mKG(N)) fused to the C-terminus of NPC1 (NPC1-mKG(N))) and 51 aa C-terminus fragment (mKG(C)) fused to the C-terminus of HAVCR1 (HAVCR1-mKG(C)). The interaction of NPC1 and HAVCR1 results in BiFC of mKG that emits peak fluorescence at 507 nm upon excitation at 492 nm. b, Huh7 NPC1 KO cells transfected with vector, plasmid coding for NPC1-mKG(N) wild type or NPC1 mutants L175A/L176A (double mutation in N-terminus D cholesterol binding pocket prevents function of NPC1), P202A/F203A (double mutation in NTD in the rim of the cholesterol binding pocket prevents function of NPC1), F503W (mutation in domain C increases binding of GP), F503Y (mutation in domain C reduces infectivity of rVSV-ZEBOV GP by 2 logs), L656F (mutation in sterol-sensing domain increases cholesterol binding, Millard 2005), G660R (Mutation transmembrane helix 3 prevents function of NPC), or P691S (mutation in transmembrane domain results in defect in cholesterol uptake and trafficking) and co-transfected or not with a plasmid coding for HAVCR1-mKG(C) for 48 h to determine HAVCR1-NPC1 interaction (green). Nuclei were stained with Hoechst 33342 (blue). Cells were analyzed in an Axiovert 200 fluorescence microscope, and micrographs were taken using a 40x objective. Scale bars represent 50 mm. Results are representative of 3 independent experiments.
Extended Data Fig. 7 | Effect of NPC1 mutations and cell entry inhibitors in exo-HAV infection. a, Huh7 NPC1 KO cells were transfected with cDNA of NPC1 WT, NPC1 mutants L173A/L176A, P202A/F203A, F503W, F503Y, L656F, G660R, or P691S, or vector and infected at 48 h p.t. with purified exo-HAV from Fig. 1c. Total RNA was extracted 4 days p.i. and analyzed by HAV RT-qPCR. Data are mean ± sem, \( n = 3 \) from 3 independent experiments. \( P \) values between NPC1 WT and NPC1 mutants or vector were determined by one-way ANOVA with Dunnett’s post-test.

b, Cell transfectants as in (a) were infected with rVSV-EBOVgp-GFP for 24 h, and virus in the supernatant was titrated by a fluorescence endpoint dilution assay in 96-well plates containing Huh7 monolayers. Viral titers were determined at 72 h p.i. using the ID50 program. Data are \( \log_{10} \text{TCID50/ml} \pm \text{s.d.} \), \( n = 3 \). \( P \) values were determined as in (a).

c, Viability of Huh7 cells treated with cell entry inhibitors for 72 h by dual-fluorescence Acridine Orange / Propidium Iodide assay. Data are mean ± sem, \( n = 4 \) from two independent experiments with biological duplicates.

d, Huh7 cells were treated with inhibitors for 1 h, infected with rVSV-EBOVgp-GFP, and GFP fluorescence was quantified at 16–20 h p.i. in a fluorescence plate reader. Data are mean ± sem, \( n = 4 \) from two independent experiments with biological duplicates.

e, Huh7 cells were pretreated with inhibitors for 1 h, infected with purified vpHAV from Fig. 1c. HAV replication was quantitated at 72 h p.i. by RT-qPCR. Data are mean ± sem, from left to right \( n = 5, 5, 3, 3 \), and 3 biological replicates. \( P \) values between DMSO and inhibitors were determined as in (a). In (c) and (d), \( P \) values between DMSO and inhibitors were determined by two-sided Mann-Whitney test.
Extended Data Fig. 8 | Chlorpromazine and Dynasore inhibit clathrin-mediated endocytosis. a, Huh7 cells were treated with inhibitors of endocytic pathways (5 µg/ml CPZ, 80 µM Dynasore, 10 µM EGA, 40 µM EIPA, 10 µM LY294002) or DMSO prior to the addition of tetrarmethylrhodamine conjugate-transferrin (Tritc-Transferrin), a marker of clathrin-mediated endocytosis. Tritc-Transferrin (red fluorescence) uptake in the presence of inhibitors was analyzed using an LSM 700 confocal fluorescence microscope. Nuclei were stained with DAPI (blue fluorescence). Micrographs were taken with a 40x oil objective. Scale bars represent 50 µm. b, Quantitative analysis of the endocytosis of Tritc-Transferrin from (a). The Tritc-Transferrin fluorescence intensity (red) of 16 cells was measured using ImageJ software, and the experiment was repeated 3 times (n=48). Box and whiskers plot was done using the Tukey method. Box limits, upper and lower quartiles; Center line, median; whiskers, 1.5x interquartile range; points, outliers. P values between DMSO and inhibitors were determined by two-sided Mann-Whitney test.
Extended Data Fig. 9 | Dynasore but not chlorpromazine inhibit caveolae-mediated endocytosis. a, Huh7 cells were treated with BODIPY FL C5-Lactoceramide complexed to BSA (LAC-cer), a marker of caveolae-mediated endocytosis, and treated with inhibitors of endocytosis (5 μg/ml CPZ, 80 μM Dynasore, 10 μM EGA, 40 μM EIPA, 10 μM LY294002) or DMSO as control. LAC-cer (green) uptake was analyzed using a LSM 700 confocal microscope. Nuclei were stained with DRAQ5 (blue). Micrographs were taken with a 40x oil objective. Scale bars represent 50 μm. b, Quantitative analysis of the endocytosis of LAC-cer from (a). The LAC-cer fluorescence intensity (green) of 20 cells was measured using ImageJ software, and the experiment was repeated 3 times (n=60). Box and whiskers plot was done using the Tukey method. Box limits, upper and lower quartiles; Center line, median; whiskers, 1.5x interquartile range; points, outliers; P values between DMSO and inhibitors were determined by two-sided Mann-Whitney test.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Effect of endocytosis inhibitors in different cellular compartments. Huh7 cells in 8-wells chamber slides were infected with the CellLight Bacmam 2.0 reagents fused to GFP (green) or RFP (red) at a multiplicity of infection of 30 particles per cell, incubated at 37°C for 10–12 h, treated with 80 mM Dyn asore hydrate, 5mg/ml Chlorpromazine hydrochloride solution, 10 mM EGA, or a similar volume of DMSO vehicle as negative control, and incubated for additional 12-14 h at 37°C. Nuclei were stained with DRAQ5 (blue), cells were fixed with 4% PFA, coverslips mounted with ProLong Gold antifade reagent, and slides analyzed in a LSM 700 confocal microscope. Micrographs were taken using a 63X oil objective. Cells were infected with CellLight Bacmam 2.0 driving the expression of markers of: a, Mitochondria (leader sequence of E1 alpha pyruvate dehydrogenase fused to RFP); b, Lysosomes (Lamp1 fused to RFP); c, Golgi (human Golgi-resident enzyme N-acetylgalactosaminyltransferase 2 fused to RFP); d, Peroxisomes (peroxisomal C-terminal targeting sequence fused to GFP); e, Early endosomes (EE, Rab5a fused to GFP); or f, Late Endosomes (LE, Rab 7a fused to GFP). Scale Bars represent 25µm. Results are representative of 3 independent experiments.
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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- A description of all covariates tested
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- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Gen5 software was used to collect absorbance and fluorescence data from the Synergy HT multimode plate reader. Cytosoft 5.3 software was used to collect data from the Guava Flow cytometer (Millipore). BD FACSDiva 8.0.1 was used to collect data from the FACSCanto II (BD Biosciences). Axiovision software version 4.8 was used to acquire fluorescence microscopy data. LSM software ZEN 2011 was used to acquire confocal microscopy data.

Data analysis

FlowJo version 8.5 and 10 were used to analyze flow cytometer data obtained with FACSCanto II (BD Biosciences) and Guava Flow cytometer (Millipore), respectively. QuantStudio Real-Time PCR Software was used to analyze RT-qPCR data. Prism 8 software (GraphPad) was used to generate graphs and to perform statistical analyses. ImageJ version 1.8.0 software was used to analyze confocal microscopy data.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Unprocessed Western blot images for ED_Fig3 and Numerical and Statistical data for Fig1, Fig2, Fig3, Fig4, ED_Fig1, ED_Fig2, ED_Fig3, ED_Fig4, ED_Fig5, ED_Fig7, ED_Fig8, ED_Fig9, and Supplementary Fig1 are provided as source data. Additional data supporting the findings of this paper will be made available from the corresponding author upon request.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample-size calculations were not performed. All experiments were done with replicates and successfully reproduced 2 or 3 times to demonstrate the magnitude and consistency of measurable differences, as is standard in the field. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | Data were not excluded. |
| Replication | All experiments were reproduced successfully 2 or 3 times. |
| Randomization | Samples were not randomized. We only analyzed one variable per experiment. |
| Blinding | We did not use externally supplied samples. Most experiments from collection to analysis were done by only one Investigator (M.I.C.), therefore blinding was not possible. |

Reporting for specific materials, systems and methods

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| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a                             | Involved in the study |
| ☐ Antibodies                    | ☐ ChIP-seq |
| ☐ Palaeontology                 | ☐ Flow cytometry |
| ☐ Animals and other organisms   | ☐ MRI-based neuroimaging |
| ☐ Clinical data                 |         |

Antibodies

- Purified mouse anti-human CD365 (HAVCR1) monoclonal antibody, clone 1D12 [BioLegend, Inc., Cat# 353902, Lot# N/A*] was used undiluted at 1 ug/test.
- PE-labeled purified mouse anti-human CD365 (HAVCR1) monoclonal antibody, clone 1D12 [ThermoFisher Scientific, Cat# 12-3659-42, Lot# N/A*] was used undiluted at 5 ul/test.
- Purified mouse anti-human CD365 (HAVCR1) monoclonal antibody, clone HAVCR1-1, developed and produced at the Kaplan’s Lab was used undiluted at 1 ug/test.
- Biotinylated affinity-purified goat anti-human TIM-1 (HAVCR1) antibody [R&D Systems, Cat# BAF1750, Lot# JVH03] was used undiluted at 1 ug/test.
- Rabbit anti-human 70-kDa heat shock protein (HSP70) polyclonal antibody [System Bioscience, Cat# EXOAB-Hsp70A-1, Lot# N/A*] was used at 1:10,000 dilution.
- Rabbit anti-human 70-kDa heat shock protein (HSP70) polyclonal antibody [System Bioscience, Cat# EXOAB-Hsp70A-1, Lot# N/A*] was used at 1:20,000 dilution.
- Mouse anti-human Flotillin-1 (FLOT-1) monoclonal antibody, clone C-2 [Santa Cruz Biotechnology, Inc., Cat# sc-74566, Lot# N/A*] was used undiluted at 0.5 ug/ml.
- Mouse anti-human tumor susceptibility gene 101 protein (TSG101) monoclonal antibody, clone 4A10 [Abcam, Cat# ab83, Lot# N/A*] was used undiluted at 0.5 ug/ml.
- Mouse anti-human golgin subfamily A member 1 (GOLGA1) protein monoclonal antibody, clone CD4 [ThermoFisher Scientific,
Cat# A-21270, Lot# N/A*) was used undiluted at 0.5 ug/ml. Affinity purified peroxidase-labeled goat anti-mouse IgG (H+L) human serum adsorbed (KPL SeraCare, Cat# 5220-0341, Lot# N/ A*) was used at 1:20,000 dilution.

Lot# N/A*: Because of the lockdown in the US due to the SARS-CoV-2 pandemic, we don’t have access to the lot numbers at the present time.

**Validation**

All commercially available antibodies were validated by the manufacturer, and the data is available in their corresponding websites.

The HAVCR1 mAb was raised at the Kaplan’s Lab against a HAVCR1-Fc fusion protein. MAb was purified by affinity chromatography in protein G columns. HAVCR1 mAb was validated at the Kaplan’s lab by reactivity against the IgV domain of HAVCR1, protection of cells against HAV infection, and blocking of binding of apoptotic cells.

Data is provided in the manuscript for the following validations:
- Anti-human HAVCR1 antibodies were validated by comparing flow cytometry results of parental cells vs HAVCR1 KO cells and vector-transfected vs HAVCR1-transfected HAVCR1 KO cells.
- Anti-human CD9, CD63, and CD81 were validated by flow cytometry with Huh7 cells and by comparison of samples with or without exosomes.
- Anti-human FLOT-1, HSP70, TSG101, and GOLGA1 were validated by comparing the Western blot results of samples with or without exosomes produced by Huh7 cells.
- Mouse IgG1 isotype control, clone 15H6 and PE-labeled anti-human CD3, clone UCHT1 were validated by flow cytometry for the lack of cell and exosome surface staining.

**Eukaryotic cell lines**

**Policy information about cell lines**

**Cell line source(s)**
- Huh7 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Cat# JCRB0403) and selected for the stable growth of wild-type HAV
- AGMK cells were obtained from the Lab of Dr. Y Moritsugu in Japan.
- Human embryonic kidney cells HEK-293 cells (Cat# 11631017, ThermoFisher Scientific)
- Vero E6 cells (Cat# CRL-1586, ATCC)

**Authentication**
- Huh7 cells were authenticated by STR profiling. Huh7 HAVCR1 KO and Huh7 NPC1 KO cells were authenticated by nucleotide sequence analysis of the KO genes. Huh7 NPC1 KO were also authenticated by resistance to EBOV cell entry.
- AGMK cells were authenticated by staining with anti-HAVCR1 monoclonal antibody 190-4, which stains a HAVCR1 antigenic variant only present in these cells (Kaplan et al., 1996; Feigelstock 1998), and sequencing of monkey HAVCR1 specific allele.
- Vero E6 and HEK-293 cells were not authenticated but cells were maintained at low passage from the original stock.

**Mycoplasma contamination**
- Huh7 and AGMK cells were mycoplasma free as assessed by h-Impact Profile II test (IDEXX BioResearch)
- The knockout cell lines and HEK293 cells were mycoplasma free as assessed by DAPI staining and fluorescence microscope analysis.
- Vero E6 cells were not tested for mycoplasma contamination.

**Commonly misidentified lines**
- No commonly misidentified cell lines were used.

**Flow Cytometry**

**Plots**
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

**Methodology**

**Sample preparation**
- Transfected or untransfected cells lines grown in cell culture flasks or plates were trypsinized and resuspended in PBS containing 2% FBS. Approximately 1,000,000 cells were treated with PE-labeled mouse monoclonal antibodies , washed, and fixed in 2% paraformaldehyde or treated with mouse monoclonal antibodies, washed, stained with PE-labeled anti-mouse secondary antibodies, washed, and fixed in 2% paraformaldehyde. Staining of the HAVCR1 mucin was performed with biotin-labeled 1750 mAb and Alexa Fluor 488-labeled streptavidin.
- Exosomes were absorbed to 2μl of 4 μm Aldehyde/Sulfate Latex Beads, 4% v/v (ThermoFisher Scientific), stained with PE-labeled monoclonal antibodies, washed, and analyzed.
- For the caspase-3 apoptosis assay, cells were trypsinized, stained with 1μl of cell-permeable caspase-3 inhibitor DEVD-FMK conjugated to sulfo-rhodamine (Red-DEVDFMK), washed, and analyzed.

**Instrument**
- Guava Flow cytometer (Millipore) and FACSCanto II (BD Biosciences)
Cytosoft 5.3 software was used to collect data from the Guava Flow cytometer (Millipore). BD FACSDiva 8.0.1 was used to collect data from the FACSCanto II (BD Biosciences). FlowJo version 8.5 and 10 were used to analyze flow cytometer data obtained with FACSCanto II (BD Biosciences) and Guava Flow cytometer (Millipore), respectively.

The population that we analyzed corresponded to 100% of the cells. We did not perform cell sorting of any other enrichment procedure.

Cells with a very low FCS/SSC profile that were close to the X- and Y-axis crossing were not included in the analysis. All other cells were included in the analysis using a wide-open gate. Boundaries between positive and negative staining cell populations were determined by using primary antibody isotype controls. Cells stained more than the isotype controls were considered as positive cells. Gating strategy is provided in the main and extended data figures next to the corresponding FACS analyses.