Virology 422 (2012) 377–385

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

# Regulation of hepatitis C virus secretion by the Hrs-dependent exosomal pathway

Keiichi Tamai [a,b], Masaaki Shiina [b], Nobuyuki Tanaka [a,d,*], Takashi Nakano [e], Akitsugu Yamamoto [f], Yasuteru Kondo [b], Eiji Kakazu [b], Jun Inoue [b], Koji Fukushima [b], Kouichi Sano [e], Yoshiyuki Ueno [b], Tooru Shimosegawa [b], Kazuo Sugamura [a,c]

[a] Division of Cancer Biology and Therapeutics, Miyagi Cancer Center Research Institute, Natori 981-1293, Japan
[b] Department of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, 980-8575, Japan
[c] Department of Microbiology and Immunology, Tohoku University Graduate School of Medicine, Sendai, 980-8575, Japan
[d] Department of Cancer Science, Tohoku University Graduate School of Medicine, Sendai, 980-8575, Japan
[e] Department of Microbiology and Infection Control, Faculty of Medicine, Osaka Medical College, Takatsuki, Osaka 569-8686, Japan
[f] Department of Animal Bio-Science, Faculty of Bio-Science, Nagahama Institute of Bio-Science and Technology, Nagahama, 526-0829, Japan

ARTICLE INFO

*Article history:*
Received 14 July 2011
Returned to author for revision
6 September 2011
Accepted 10 November 2011
Available online 3 December 2011

*Keywords:*
Hrs
ESCRT
HCV

ABSTRACT

The molecular mechanisms of assembly and budding of hepatitis C virus (HCV) remain poorly understood. The budding of several enveloped viruses requires an endosomal sorting complex required for transport (ESCRT), which is part of the cellular machinery used to form multivesicular bodies (MVBs). Here, we demonstrated that Hrs, an ESCRT-0 component, is critical for the budding of HCV through the exosomal secretion pathway. Hrs depletion caused reduced exosome production, which paralleled with the decrease of HCV replication in the host cell, and that in the culture supernatant. Sucrose-density gradient separation of the culture supernatant of HCV-infected cells revealed the co-existence of HCV core proteins and the exosome marker. Furthermore, both the core protein and an envelope protein of HCV were detected in the intraluminal vesicles of MVBs. These results suggested that HCV secretion from host cells requires Hrs-dependent exosomal pathway in which the viral assembly is also involved.



## Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease, with about 170 million people infected worldwide. It is important to identify the molecular basis of HCV infection, propagation, and pathogenesis in humans. HCV is a positive-strand RNA virus belonging to the *Flaviviridae* family and the sole member of the genus *Hepacivirus*. The HCV RNA genome serves as a template for viral replication and as a messenger RNA for viral production. It is translated into a single immature polyprotein of approximately ~3000 amino acids that is further cleaved into at least 10 mature proteins by proteases (Asselah et al., 2009).

The HCV life cycle starts with virion attachment, especially through its envelope glycoproteins E1 and E2, to specific receptors on the host cells, which include CD81, claudin-1, and the class B member I scavenger receptor (Evans et al., 2007; Murray et al., 2008). These receptors and co-receptors are also required for HCV entry, which involves an additional clathrin-mediated post-internalization step and delivery into early endosomes (Burlone and Budkowska, 2009). Recently, the complete replication of HCV in a cell culture system was achieved (Wakita et al., 2005). Nevertheless, little is known about how the assembled HCV virion is released from the cytoplasm.

Extensive research on human immunodeficiency virus (HIV) has shown that the ESCRT (endosomal sorting complex required for transport) machinery plays a crucial role in virion assembly and budding from cellular membranes: The late-domain (L-domain) motif of HIV-Gag binds TSG101 (an ESCRT-I component) and Alix (a protein that bridges ESCRT-I and ESCRT-III). Virion assembly, budding, and release are subsequently achieved with help from the sequential downstream ESCRT machinery (Chen and Lamb, 2008). Other enveloped viruses, such as hepatitis B virus and human T-cell leukemia virus type I, also hijack the cellular membrane trafficking and sorting networks to accumulate and assemble their viral components, after which the nascent virions pinch themselves off for release (Chen and Lamb, 2008).

Similar to an enveloped virus particle that buds from the host-cell surface, small vesicles, called exosomes, are physiologically secreted from a variety of cells (Denzer et al., 2000; van Niel et al., 2006). Exosomes are nanovesicles (60–90 nm in diameter) surrounded by a

*Abbreviations:* ER, endoplasmic reticulum; ESCRT, endosomal sorting complex required for transport; HCV, hepatitis C virus; HHV-6, human herpes virus-6; HIV, human immunodeficiency virus; ILV, intraluminal vesicle L-domain, late-domain; MVB, multivesicular body; shRNA, short hairpin RNA.

* Corresponding author at: Division of Cancer Biology and Therapeutics, Miyagi Cancer Center Research Institute, 47-1 Nodayama, Medeshima-Shiode, Natori, Miyagi, 981-1293, Japan. Fax: +81 22 381 1168.
*E-mail address:* tanaka@med.tohoku.ac.jp (N. Tanaka).

0042-6822/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2011.11.009

lipid bilayer. They are generated as intraluminal vesicles (ILVs) within a sorting endosome called a multivesicular body (MVB), by the inward budding of the MVB's limiting membrane. Exosome release into the extracellular milieu is achieved by direct fusion of the MVB with the plasma membrane (Pan et al., 1985).

The sorting of ubiquitinated proteins on MVBs is mediated by the ESCRT pathway. The first complex that binds the cargo on endosomes is ESCRT-0 which includes Hrs (Asao et al., 1997; Komada and Kitamura, 1995) and STAMs, and with the help of ESCRTs-I, -II, and -III, the cargo accumulates on the endosomal membrane. At the end

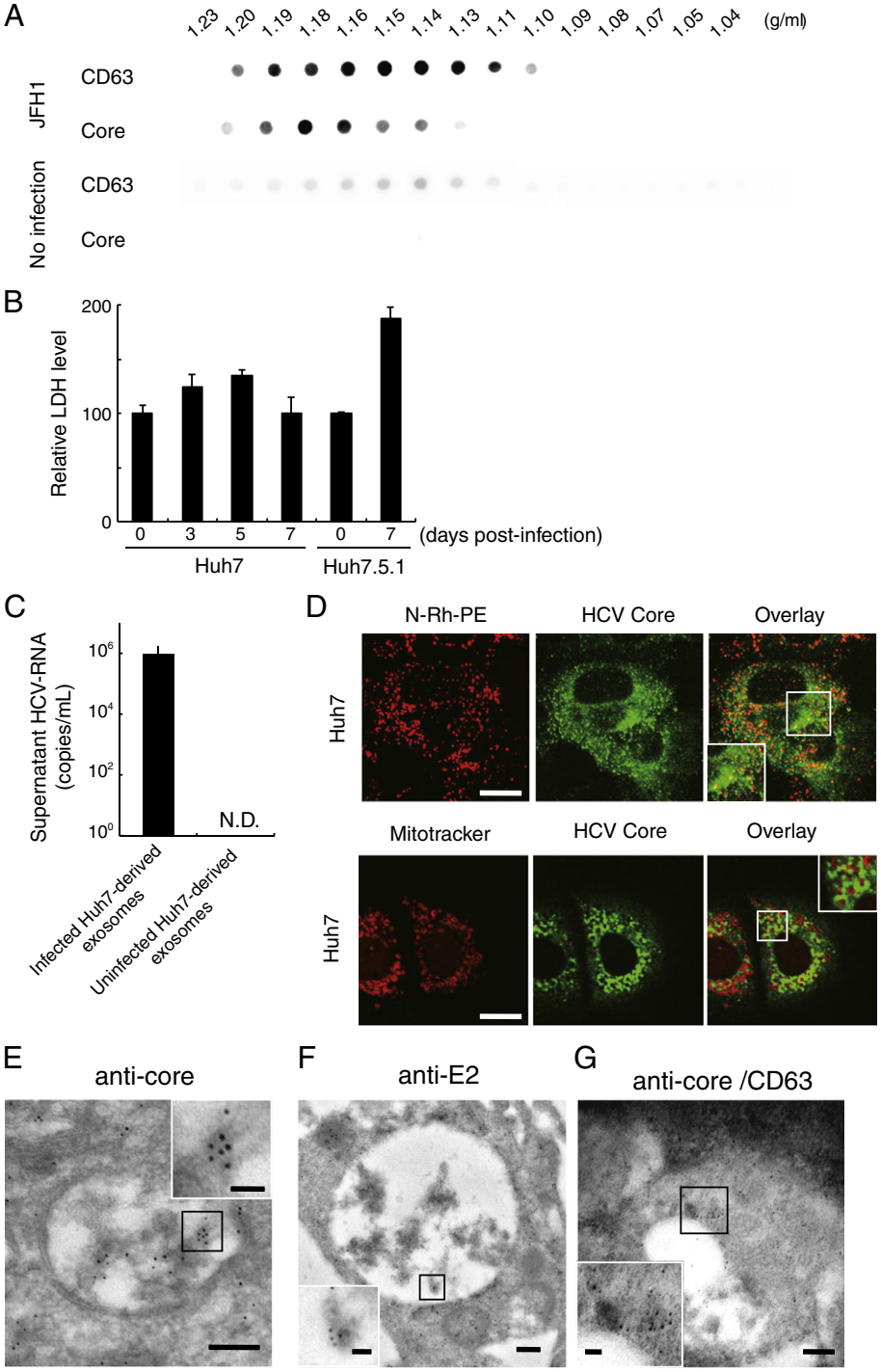


**Fig. 1.** Localization of HCV protein to exosome-rich fractions. (A) Dot blot analysis of the supernatants of JFH-1-infected Huh7 cells fractionated on a sucrose gradient and examined for an exosome protein (CD63) and HCV core protein. Huh7 cells were uninfected or infected with JFH-1 (MOI = 0.01), and the medium was changed after 3 days. The cells were then incubated for 2 days, and the culture supernatants were harvested and purified by ultracentrifugations. (B) Huh7 cells were infected at a multiplicity of infection of 0.01 with HCV and Lactate dehydrogenase was quantitated in culture supernatants. (C) HCV-RNA in the culture supernatant at day 5 post-infection after infection with purified exosomes from infected and uninfected Huh7 cells, measured using real-time PCR. Purified exosomes were obtained from 10 ml of supernatant of Huh7 cells at day 5 post-infection. N.D., not detectable. (D) Upper panel: Confocal microscopic image of Huh7 cells infected with JFH-1. The exosome and MVB marker, N-Rh-PE (red) and the HCV core protein (green) were partially colocalized. Lower panel: anti-core protein (green) and Mitotracker (red) were used. Bar, 20 μm. (E and F) Immunoelectron microscopy of an MVB in wild-type Huh7 cells. Sections of Huh7 cells were incubated with antibodies against HCV core protein (5-nm-colloidal-immunogold-labeled anti-mouse goat IgG in E) and HCV envelope protein E2 (5-nm in F). Insets show a higher magnification. Bars: 200 nm, bars in insets: 50 nm. (G) Double-staining of immunoelectron microscopy using antibodies against HCV core protein (10 nm) and anti-CD63 (5 nm). Insets show a higher magnification. Bars: 200 nm, bars in insets: 50 nm.

of sorting, an AAA-type ATPase, VPS4, disrupts the ESCRT complexes, and the membrane with its accumulated cargo is invaginated into the maturing endosome to produce an MVB. A deficiency of Hrs results in abnormally enlarged endosomes and a marked reduction in cargo sorting to MVBs. The enlarged endosomes accumulate ligand-activated membrane-bound growth factor receptors (Tanaka et al., 2008). In this context, we recently demonstrated that Hrs is also required in dendritic cells for exosome secretion and antigen presentation via exosomes (Tamai et al., 2010).

Relationships are also reported between membrane trafficking and the HCV life cycle. ESCRT-III contributes to HCV release in Huh7 cells (Ariumi et al., 2011; Corless et al., 2010), as does transfection of these cells with RNAi against Atg7, an essential autophagy gene (Tanida et al., 2009). Another study suggested that human plasma contains exosomes and that in HCV patients, viral RNA is associated with these circulating vesicle (Masciopinto et al., 2004). Since Hrs, an ESCRT-0 component, is involved in the autophagic pathway (Tamai et al., 2007) and required for exosome secretion, we hypothesized that Hrs plays a role in the HCV life cycle. Here we demonstrated that the Hrs-dependent exosomal pathway plays an important role in HCV release.

## Results

### *HCV core protein localizes to exosome-rich fractions*

A relationship between virus production and the exosome pathway has been suggested for HIV and human herpes virus 6 (HHV6) (Lenassi et al.; Mori et al., 2008). We suspected that HCV also utilizes the exosome pathway for its release from host cells. To examine this possibility, we subjected the culture supernatants of HCV JFH-1-infected Huh7 cells to sucrose density gradient centrifugation and analyzed the fractions for exosomes and HCV. Interestingly, the identical fractions were positive both for the exosome marker CD63 and for the HCV core protein (Fig. 1A). Notably, more CD63-positive exosomes were found in the HCV-infected Huh7 cells than in uninfected cells. To exclude a possibility that the cytopathic effect (CPE) induced by the infection of JFH-1 affects the amount of CD63 in the supernatant, we monitored the CPE by the lactate dehydrogenase (LDH) assay. In Huh7 cells, we observed slight increase of LDH at post-infection day 5 (Fig. 1B). As a positive control, we also investigated the CPE in Huh7.5.1, which is more susceptible to JFH-1 infection. LDH level was approximately two-times higher in this Huh7.5.1 cells than the non-infected, which is compatible with a previous report (Shiina and Rehermann, 2008). Purified exosomes from the culture supernatant of JFH-1-infected Huh7 cells possessed HCV-infectivity to Huh7 cells (Fig. 1C). These results indicated that the exosome secretion was mainly activated during HCV infection.

We next addressed whether HCV localizes to the intraluminal vesicles within the MVBs of JFH-1-infected Huh7 cells. Using confocal microscopy, we found that N-Rh-PE-positive dots (an exosome and MVB marker) were likely to colocalize with HCV-core-positive dots (Fig. 1D, upper panels). As a negative control, immunofluorescence staining was performed with anti-HCV-core and mitochondrial marker (Mitotracker (Molecular Probes)), which reportedly do not colocalize with each other (Lai et al., 2010). We did observe that HCV-core protein was not colocalized to mitochondria (Fig. 1D, lower panels). By immunoelectron microscopy, we detected positive immunogold staining for the HCV core antigen on the intraluminal vesicles (Fig. 1E). Similarly, positive immunogold staining for the HCV envelope protein E2 was observed on intraluminal vesicles within MVBs (Fig. 1F). To make the relationships between HCV particles and exosomes clearer, we performed the double-staining of immunoelectron microscopy using anti-CD63 and anti-core protein. We observed the colocalization of CD63 and HCV-core in the intraluminal vesicles in the MVB (Fig. 1G). These data together suggested that HCV virions and exosomes are present in the same or very similar compartments.

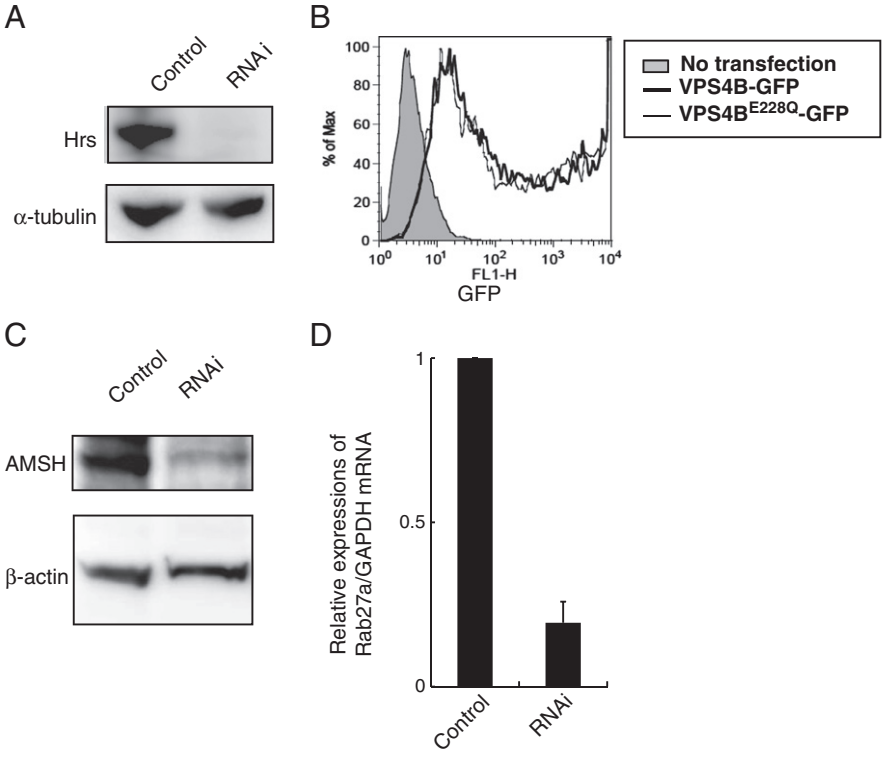


**Fig. 2.** Establishment of Huh7 lines with knocked-down Hrs, AMSH or Rab27a, and overexpressed VPS4B. (A) Western blot analysis of total-cell lysates from control and Hrs (A) and AMSH (C) knock-down Huh7 cells. (B) Flow-cytometry analysis of Huh7 cells overexpressing GFP-fused VPS4B (wild-type and dominant-negative (E228Q) forms). (D) Quantitative real-time PCR analysis of Rab27a in Huh7 cells. Rab27a mRNA expression was normalized to GAPDH.

*Hrs plays crucial roles in HCV and exosomal secretion*

Since Hrs is to be required for exosome secretion (Tamai et al., 2010), we examined the roles of membrane traffic-related proteins in HCV secretion. For this study, we established an Hrs-depleted Huh7 cell line using shRNA against human Hrs (Fig. 2A), and Huh7 cell lines that overexpressed wild-type VPS4B, and an ATPase-negative VPS4B (VPS4B$^{E228Q}$, Fig. 2B). We also prepared knock-down Huh7 cell lines for AMSH, a deubiquitination enzyme that binds the ESCRT-III complex, and for Rab27a, a Rab GTPase required for exosome secretion, using specific shRNAs (Figs. 2C and D).

First, we confirmed that Hrs plays a crucial role in exosome secretion in Huh7 cells. Exosome secretion of the control Huh7 cells was clearly increased by 48 h of stimulation with $Ca^{2+}$ ionophore A23187, a well-established stimulator for exosome secretion (Savina et al., 2005), whereas a smaller increase was observed in the Hrs-depleted Huh7 cells (Fig. 3). These data confirmed Hrs's crucial role in the exosome secretion of Huh7 cells.

Next, using the various Huh7 cell lines, we analyzed whether membrane-traffic-related proteins are involved in HCV release. We first compared the amount of HCV-RNA in the culture supernatants of the Huh7 cell lines. The amount of HCV-RNA was significantly lower in the supernatant of the Hrs-depleted cells from days 3 to 5 post-infection (Fig. 4A). Similarly, overexpression of the dominant-negative VPS4B decreased the HCV-RNA production in Huh7 cells (Fig. 4B), consistent with a previous report (Corless et al., 2010). In contrast, there was no difference in the HCV release between the AMSH-depleted and control Huh7 cells (Fig. 4C). However, HCV-RNA was dramatically decreased in the Rab27a-depleted Huh7 cells (Fig. 4D).

We also confirmed the amount of HCV by measuring the HCV infectious titer in the supernatant. The virus titer was significantly decreased in the absence of Hrs, Rab27a and overexpressed ATPase defective-VPS4B, but not in the absence of AMSH (Fig. 4E). These data were compatible with the amount of HCV-RNA.

Since Hrs-depletion decreased the Huh7 cells' growth rate (data not shown), we also examined the amount of HCV using X-ray-irradiated cells to inhibit the cell proliferation, and eliminate the cell growth effect. In a preliminary experiment, we confirmed that irradiation with X-rays (25 Gy) was enough to inhibit the Huh7 cell proliferation completely (data not shown). The amount of HCV-RNA in the supernatant was decreased in the Hrs-depleted irradiated Huh7 cells compared with control cells (Fig. 5). These data together indicated that Hrs plays a crucial role in HCV secretion.

*Analysis of intracellular HCV-RNA*

We next assessed whether the decreased HCV release observed in the Huh7 cell lines reflected a decrease in viral reproduction, by examining the intracellular HCV-RNA. In the Hrs-depleted Huh7 cells, no significant decrease in the amount of intracellular HCV-RNA was found (Fig. 6A). Similar results were obtained in the Huh7 cells expressing dominant-negative VPS4B and those with AMSH-depletion (Figs. 6B and C). In the Rab27a-depleted Huh7 cells, the amount of intracellular HCV-RNA was decreased on day 5. (Fig. 6D).

Next, we quantified the amount of intracellular HCV core protein using Western blot analysis. The expression of core protein was almost the same between in the Hrs-depleted Huh7 cells and the control cells on days 5 and 7 post-infection (Fig. 6E). These results suggested that Hrs is not involved in HCV replication or translation.

Since the HCV-RNA in the supernatant was decreased in Hrs-depleted Huh7 cells without any decrease in the intracellular HCV-RNA or core protein, the assembly and/or release of HCV might be impaired in the absence of Hrs. Hence, we examined the effect of Hrs depletion on the amount of intracellular infectious HCV particles by performing a focus-forming unit assay. Intracellular infectious HCV particles were decreased in Huh7 cells with Hrs-depletion and Rab27a-depletion and those expressing dominant-negative VPS4B compared with control cells on day 5 post-infection (Figs. 6F to I). These data suggested that Hrs is required at least for HCV assembly.

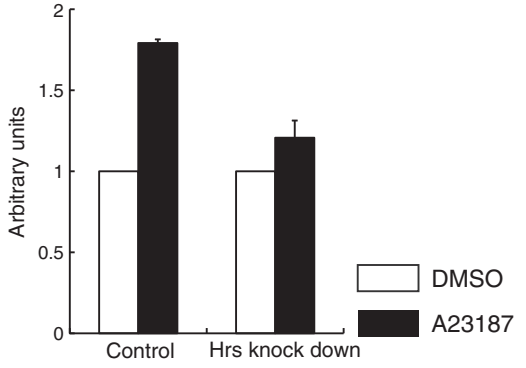


**Fig. 3.** Hrs affects exosome release in Huh7 cells. Measurement of exosomes under stimulation with a $Ca^{2+}$ ionophore (A23187), using a protein assay. Control and Hrs-knock-down Huh7 cells were incubated with 1 μM A23187 for 48 h.

## Discussion

This study addressed the relationship between the ESCRT machinery and HCV viral release. Although the initial studies on ESCRT proteins mostly focused on their role in the endosomal sorting and lysosomal digestion of membrane-bound receptors, there is accumulating evidence that they also play important roles in MVB formation (Wollert and Hurley, 2010). ESCRT executes scission of the endosomal limiting membranes to form the intraluminal vesicles that characterize MVBs and of the plasma membrane to release intraluminal microvesicles into the extracellular environment as exosomes (de Gassart et al., 2004). Here we demonstrated that an ESCRT-0 component, Hrs, is crucial for HCV release through the exosome secretion pathway.

Previous reports suggested that Hrs recruits ESCRT-I complex components, including Tsg101, VPS28, and VPS37, and initiates the ESCRT pathway. The ESCRT-I component Tsg101 was also reported to form a complex with HIV-1 Gag, which promotes an Hrs-independent ESCRT mechanism for HIV release (Pornillos et al., 2003). Similarly, Tsg101 is involved in the release of human T-cell leukemia virus (HTLV)-I, because Tsg101 depletion inhibits HTLV-I budding (Blot et al., 2004). These viruses utilize not only ESCRT-I but also its downstream machinery, including ESCRT-III and VPS4. This is also the case for HCV, because ESCRT proteins such as ESCRT-III, VPS4, TSG101, and Alix contribute to HCV release (Ariumi et al., 2011; Corless et al., 2010). In this context, VPS4 is one of the last and most potent molecules in the ESCRT pathway, and the dependence of enveloped-virus budding on the ESCRT machinery has been proved in several cases by showing that the budding is VPS4-dependent. Taking all these data together, we concluded that HCV takes advantage of the entire ESCRT machinery for its release.

In this study, Hrs depletion attenuated both HCV release into the supernatant and the amount of infectious particles in the cytoplasm, but not the amount of HCV-RNA in the cytoplasm, suggesting that Hrs is involved in HCV assembly. A previous study suggested that the formation of HCV virions is closely connected to lipid droplets and the endoplasmic reticulum (ER) (Miyanari et al., 2007). Here we observed two HCV antigens, the core protein and E2, within MVBs in Huh7 cells, indicating that HCV probably takes advantage of the MVBs themselves for its assembly. In this study, AMSH, which does not affect MVB formation (data not shown), was not required for HCV release, supporting our hypothesis. The relationship between the ER and MVBs is not fully understood, but a recent study suggested that direct membrane contact occurs between them (Eden et al.,

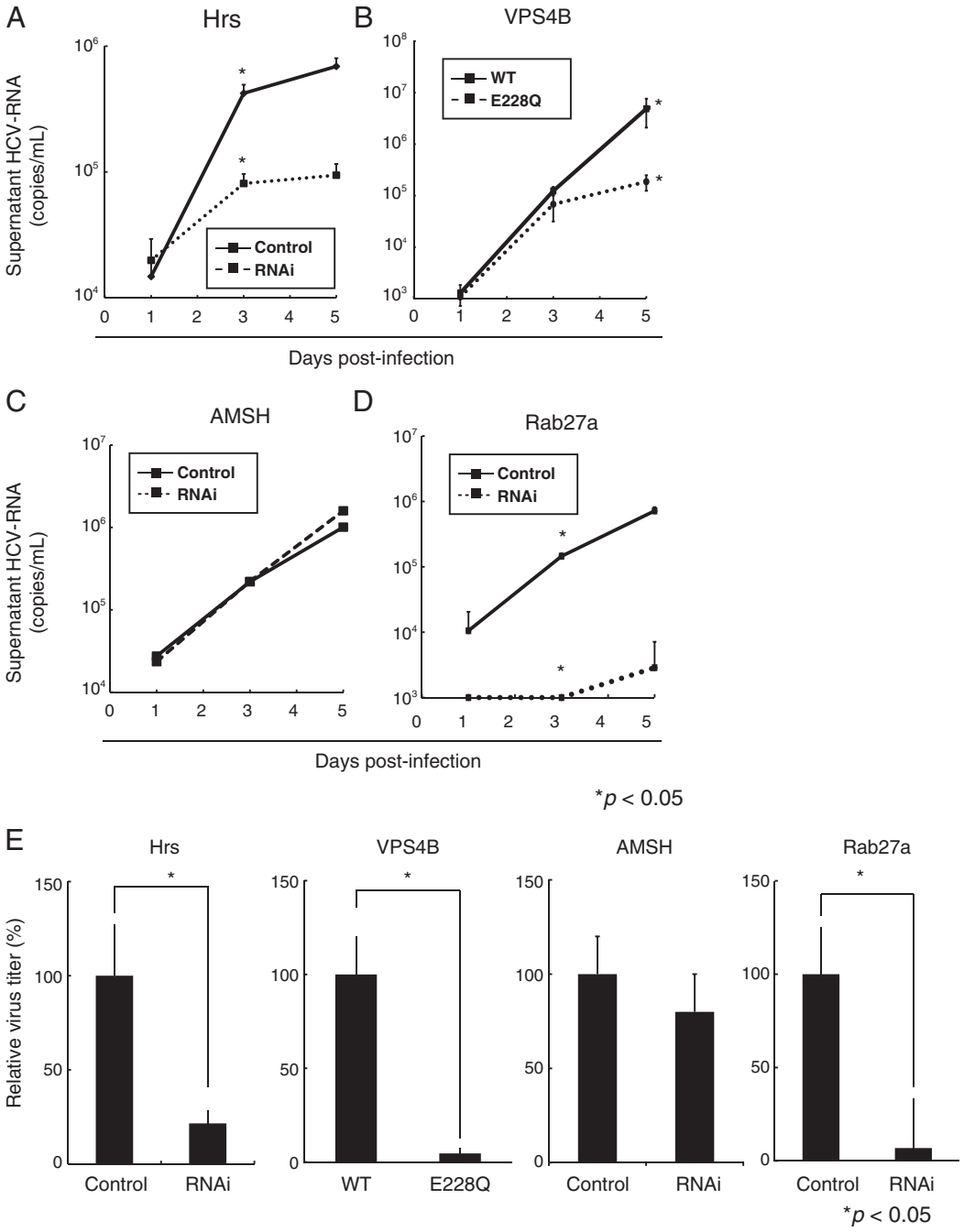


**Fig. 4.** Hrs, VPS4B, and Rab27a, but not AMSH, affect HCV release. (A to D) HCV-RNA in the culture supernatant on the indicated day after infection, measured using real-time PCR. Huh7 cells were infected with HCV (MOI = 0.01). (E) Infectious HCV particles in the supernatant were measured by a focus-forming unit assay at day 5 post-infection.

2010). Although the detailed mechanisms are not fully elucidated, we propose that normal trafficking via MVBs is required for the formation of complete HCV particles.

In this study we found the relationship between HCV and exosome from three experiments. First, by using immunoelectron microscopy, we demonstrated that HCV core protein and CD63 colocalized each other in MVB. Second, the confocal microscopy analyses revealed that exosomal marker N-Rh-PE colocalized with the core protein. Third, purified exosomal fraction possessed infectivity to Huh7 cells. These results suggest that HCV particles indeed colocalize with exosomes, and contain exosome marker proteins. This notion is not surprising because various viruses have been shown to acquire parts of host cell-derived proteins (Kolegraff et al., 2006). These phenomenons seem to reflect a fact that a number of virus carry host factors either during the assembly, or the release from their respective host cells. In Fig. 1A, the peaks for the HCV core protein and CD63 do not entirely correspond (1.18 g/ml and 1.15 g/ml, respectively). In general, HCV particles and exosomes in diameter are very similar (50 nm and 30–100 nm, respectively). On the other hand, HCV particles contain genomes which consist of a positive-sense RNA molecule approximately 9.6 kb in length. It is possible that the density of HCV particles is somewhat higher than that of exosomes. Further study will be required to elucidate how HCV particles and exosomes were similarly or differentially produced in MVB.

The detailed mechanisms by which HCV utilizes the ESCRT machinery for its release are still unknown. Capsid proteins of several viruses, such as HIV and HTLV-1 interact with ESCRT proteins via the

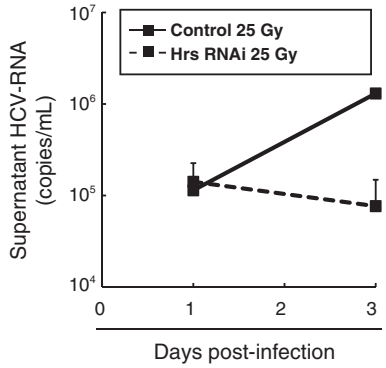


**Fig. 5.** Hrs affects HCV release in irradiated Huh7 cells. Control or Hrs knock-down Huh7 cells were irradiated with X-rays (25 Gy) and infected with HCV (MOI = 0.1). The results are the mean and SD from three parallel samples.

capsid-proteins' L-domains. This interaction recruits ESCRT to the site of capsid accumulation, and contributes to the budding of capsid-captured membranes, scission, and the release of virions, using similar mechanisms to those involved in MVB formation (McDonald and Martin-Serrano, 2009). Therefore, it is tempting to speculate a similar viral structure-ESCRT interaction scenario for HCV release. However, we found no canonical L-domains such as PT/SAP, YPXL, PPXY, or LYPXnL, within the amino acid sequence of HCV proteins. In the case of HIV-1, a previous report suggested that the HIV-1 Gag proteins are ubiquitinated and that this ubiquitination is required for HIV-1 release (Gottwein and Krausslich, 2005). Concordantly, the HCV core protein is reported to be ubiquitinated by E6AP (Shirakura et al., 2007). Thus, the ESCRTs might interact with viral proteins through the ESCRT proteins' ubiquitin-binding domain. Because Hrs possesses two ubiquitin-binding regions, UIM and VHS, Hrs may recognize the ubiquitinated HCV core proteins and sort them to the viral budding site in the MVBs, thereby enabling virion release.

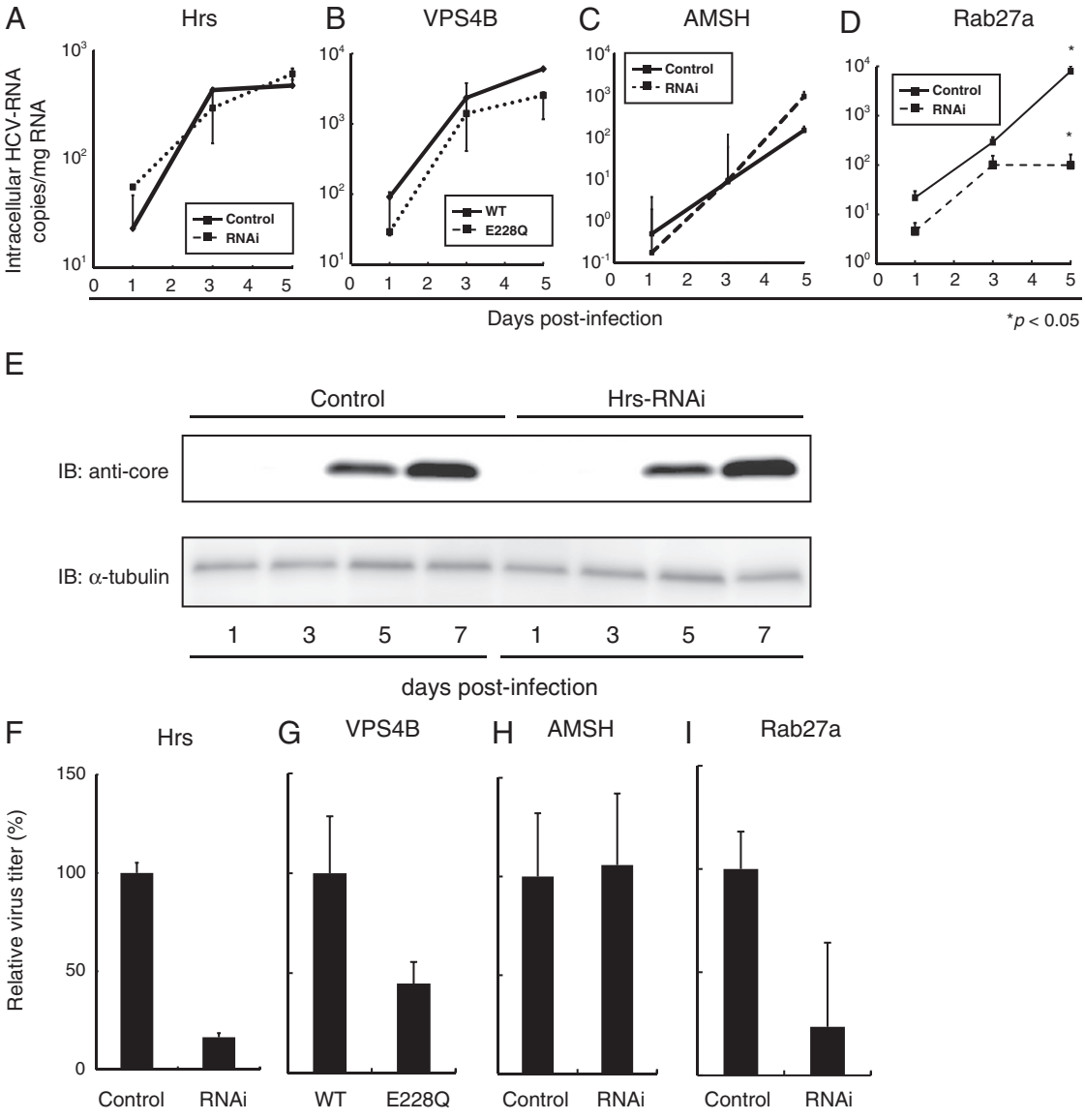


**Fig. 6.** No alteration in HCV-RNA replication in Hrs-depleted cells. (A to D) Intracellular HCV-RNA was measured in various Huh7 cell lines on the indicated day after infection. Values were normalized to the total RNA. The results are the mean and SD from three parallel samples. In VPS4B-expressing Huh7 cells, the difference is not significant through days 1 to 5. (E) Western blot analysis of HCV core protein. Total lysates were prepared from control and Hrs-depleted Huh7 cells on the indicated day post-infection and analyzed by immunoblotting with an anti-core antibody. Data are representative of three independent experiments. (F to I) Intracellular infectious HCV particles were measured by a focus-forming unit assay at day 5 post-infection.

A previous study suggested that human herpes virus-6 (HHV-6) is released together with internal vesicles through MVBs by the cellular exosomal pathway: HHV-6 undergoes maturation in the trans-Golgi network (TGN) and post-TGN-derived vacuoles, which express an exosomal marker, CD63, and MVBs contain HHV-6 envelope glycoproteins along with CD63 (Mori et al., 2008). In addition, HIV virions released into the culture supernatant also contain CD63 (Gould et al., 2003), and CD63-positive exosomes derived from HIV-infected cells contain HIV Gag proteins (Booth et al., 2006). Here we showed that CD63-positive exosomal fractions can also contain HCV particles. Taken together, these observations support the idea that exosomal secretion and the budding of HCV, HHV6, and HIV share overlapping pathways.

## Conclusion

Since we here provide evidence that Hrs is important in exosome secretion and HCV release, Hrs may be one of the key players in HCV release, through the exosomal pathway. Controlling this pathway could become one of novel therapeutic strategies for chronic viral infections.

## Materials and methods

### *Ethics statements*

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the research committees of the Miyagi Cancer Center and Tohoku University.

### *Cells*

Huh7 and Huh7.5.1 (gift from Dr. Chisari, The Scripps Research Institute, CA) cells were maintained in Dulbecco's modified essential medium containing 10% fetal calf serum and antibiotics. To express Hrs-specific short hairpin RNA (shRNA), a retroviral vector was generated as described previously (Tamai et al., 2007). In brief, a sequence encoding human Hrs-specific shRNA, was inserted into pSIREN-RetroQ (BD Biosciences) to make pSIREN-RetroQ-Hrs. The target sequence consisted of nucleotides 302–320 (5′-AGGTAAACGTCCGTAACAA-3′) of the human hrs cDNA. A control plasmid, pSIREN-RetroQ-Luc, targeted bp 413–434 of firefly luciferase (5′-GCAATAGTTCACGCTGAAAAG-3′). The retrovirus was prepared as previously described (Tamai et al., 2007). A human AMSH-specific short hairpin RNA (shRNA), pSIREN-RetroQ-AMSH, was also generated using pSIREN-RetroQ (Kyuuma et al., 2007). The target sequence within the human AMSH cDNA was nucleotides 651–669 (5′-GCAGCAATTGGAACAGGAA-3′). A human Rab27a-specific shRNA lentiviral vector was purchased from Sigma. LDH assay was performed according to the manufacturer's protocol (Roche, Basel, Switzerland).

### *Isolation and purification of exosomes*

Exosomes were purified as previously described (Ostrowski et al., 2010). In brief, the cell culture medium was centrifuged for 10 min at 300×*g*, 10 min at 1200×*g*, and 30 min at 10,000×*g* to remove cells and debris. The supernatant obtained from the last spin was then centrifuged for 60 min at 100,000×*g*, and the pellet was solubilized in lysis buffer (1% Nonidet P-40, 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin) and analyzed by BCA protein assay (Pierce, Rockford, IL, USA), according to the manufacturer's protocol. For sucrose density gradient centrifugation, sucrose gradients were prepared according to a former procedure (Abe and Davies, 1986). In brief, 5 ml 72% sucrose/PBS was overlaid with 5 ml 8.4% sucrose/PBS in 10 ml tubes, and the tubes stoppered and kept horizontal for 3 h at room temperature, after which they were slowly returned to the upright position. The exosomal suspension was layered onto a gradient sucrose and centrifuged at 100,000×*g* for 20 h in a Beckmann SW41Ti rotor.

### *Western blotting*

Immunoblotting was performed as described previously (Takeuchi et al., 1999). In brief, total cell lysates were prepared with NP-40-containing lysis buffer (described above). The lysates were pre-cleared by centrifugation (10,000×*g*) for 20 min at 4 °C, and the supernatants were separated by SDS-PAGE and transferred onto polyvinyl difluoride (PVDF) membranes (Millipore). After being blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20, the membranes were probed with the indicated primary antibodies. After another wash, the membranes were probed with HRP-conjugated secondary antibodies (Cell Signaling). For dot-blot analyses, cell lysates were spotted onto PVDF membranes. The membranes were blocked as described above and incubated with the indicated antibodies.

### *Generation and quantification of cell culture HCV RNA and infectious titer*

The HCV strain JFH-1 was a gift from Dr. T. Wakita (National Institute of Infectious Diseases, Japan) (Wakita et al., 2005). Cell-culture-derived, infectious HCV was generated as described previously (Wakita et al., 2005). The HCV was quantified as follows: RNA was extracted from the Huh7 culture supernatant using the QIAamp Viral RNA Kit (Qiagen, Valencia, CA). The HCV RNA was quantified by real-time reverse transcription polymerase chain reaction using TaqMan EZ RT-PCR Core Reagents (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol, using the published primers and probe (Takeuchi et al., 1999). The filtered (0.45 μm) culture supernatant of HCV-infected Huh-7.5.1 cells containing $2\times10^8$ HCV RNA copies/ml (equivalent to $9.7\times10^4$ focus-forming units [ffu]/ml) was used for experiments. To analyze HCV-RNA in the supernatant, Huh7 cells ($2\times10^5$ cells in 6-well plate) were infected with JFH-1 (multiplicity of infection, MOI = 0.01), washed with PBS twice after 4 h, the supernatants were collected, and the cells were reseeded at $2\times10^5$ cells per 6-well plate at the indicated times. Huh7 cells were irradiated with X-rays (25 Gy) before infection to exclude the effect of cell proliferation in some experiments. To quantify the intracellular HCV titer, infected Huh7 cells were harvested at the indicated times and lysed by three freeze-thaw cycles. The virus titer was determined by focus-forming unit assay, as previously described (Kato et al., 2006).

### *Antibodies for immunoelectron microscopy*

An anti-HCV-core mouse monoclonal antibody (mAb) (2H9, a gift from Dr. Wakita, National Institute of Infectious Diseases, Japan) and a human anti-E2 mAb (CBH5, a gift from Dr. Foung, Department of Pathology, Stanford School of Medicine) were used as primary antibodies. As secondary antibodies for immunoelectron microscopy, 5-nm-colloidal-immunogold-labeled anti-mouse goat IgG and 5-nm-immunogold-labeled anti-human goat IgG antibodies (EY Laboratories, San Mateo, CA) were used.

### *Quantitative real-time polymerase chain reaction*

To confirm the knock-down of Rab27a, quantitative real-time PCR was performed. Total RNA was prepared from cells with the RNeasy Mini kit (Qiagen). Complementary deoxyribonucleic acid (cDNA) was synthesized using an oligo d(T)12–18 primer with a Primescript reverse transcriptase (Takara Bio Inc, Japan) and subjected to quantitative real-

time PCR using a LightCycler 480 and the SYBR Green I Master kit (Roche Diagnostics). The primer sequences were, for GAPDH, 5′-CTCTGCTCCTCCTGTTCGAC-3′ and 5′-GACAAGCTTCCCGTTCTCAG-3′; and for Rab27a, 5′-TGGAGGACCAGAGAGTAGTGAAA-3′ and 5′-AGTTTCAAAGTAGGGGATTCCA-3′.

### *Phenotypic analysis of cells by flow cytometry*

Cells were assessed for VPS4B-GFP expression by fluorescent multicolor flow cytometry (FACSCantoII, Becton Dickinson, San Jose, CA) as previously described (Tamai et al., 2010).

### *Immunoelectron microscopy*

To analyze the localization of the HCV antigen in MVBs, Huh7 cells infected with JFH-1 were fixed with 1% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.2) at 4 °C for 2 h. The fixed cells were washed six times with PBS and dehydrated in a graded ethanol series. The samples were then embedded in Lowicryl K4M resin (Polyscience, Warrington, PA, USA), and allowed to polymerize in a UV irradiator (DOSAKA EM, Kyoto, Japan) at −20 °C for 2 days and then at room temperature for 2 days. Ultrathin sections were cut using an ultramicrotome (Sorvall MT-5000, Du Pont, CT, USA) and mounted on a nickel grid (300 mesh) supported by a carbon-coated collodion film. The ultrathin sections on the grid were treated with 5% normal goat serum in PBS to block non-specific reactions. The sections were then reacted with drops of primary antibody at 4 °C for 8 h, washed with PBS, reacted with secondary antibody for 1 h at room temperature, and washed again with PBS. The immunostained sections were fixed in 1% glutaraldehyde in PBS for 15 min and washed three times with distilled water. Finally, the sections were subjected to contrast-enhanced staining with 0.01% ruthenium red (Wako Pure Chemicals, Osaka, Japan) and 0.5% osmium tetroxide in PBS at room temperature for 10 min, further with 4%(w/v) uranyl acetate in distilled water for 15 min at room temperature, and observed under a transmission electron microscope (H-7650, Hitachi, Tokyo, Japan).

For double-staining of immunoelectron microscopy, post-embedding gold was carried out as described previously (Iwata et al., 1995). Mouse anti-HCV-core IgG (2H9, diluted 50×) and rabbit anti-CD63 IgG (sc-15363 Santa Cruz, diluted ×5) were used.

## Acknowledgments

The authors thank Dr. Takaji Wakita for providing JFH-1 strains and Dr. Frank Chisari for providing Huh7.5.1 cell line. The authors also thank Yoshihiko Fujioka, Chika Sasaki, Kyoko Oyama, Nozomi Endo, and Mao Nakamura for technical assistance. This work was supported by JSPS and MEXT KAKENHI (21590517, 19059001, and 22790630) and a Grant-in-Aid from the Ministry of Health, Labour and Welfare, and Takeda Medical Research Foundation.

## References

Abe, S., Davies, E., 1986. Quantitative Analysis of Polysome Profiles Using a Baseline from Uncentrifuged Blank Gradients, 31. Memories of the College of Agriculture, Ehime University, pp. 187–199.
Ariumi, Y., Kuroki, M., Maki, M., Ikeda, M., Dansako, H., Wakita, T., Kato, N., 2011. The ESCRT system is required for hepatitis C virus production. PLoS One 6, e14517.
Asao, H., Sasaki, Y., Arita, T., Tanaka, N., Endo, K., Kasai, H., Takeshita, T., Endo, Y., Fujita, T., Sugamura, K., 1997. Hrs is associated with STAM, a signal-transducing adaptor molecule. Its suppressive effect on cytokine-induced cell growth. J. Biol. Chem. 272, 32785–32791.
Asselah, T., Benhamou, Y., Marcellin, P., 2009. Protease and polymerase inhibitors for the treatment of hepatitis C. Liver Int. 29 (Suppl. 1), 57–67.
Blot, V., Perugi, F., Gay, B., Prevost, M.C., Briant, L., Tangy, F., Abriel, H., Staub, O., Dokhelar, M.C., Pique, C., 2004. Nedd4.1-mediated ubiquitination and subsequent recruitment of Tsg101 ensure HTLV-1 Gag trafficking towards the multivesicular body pathway prior to virus budding. J. Cell Sci. 117, 2357–2367.
Booth, A.M., Fang, Y., Fallon, J.K., Yang, J.M., Hildreth, J.E., Gould, S.J., 2006. Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane. J. Cell Biol. 172, 923–935.
Burlone, M.E., Budkowska, A., 2009. Hepatitis C virus cell entry: role of lipoproteins and cellular receptors. J. Gen. Virol. 90, 1055–1070.
Chen, B.J., Lamb, R.A., 2008. Mechanisms for enveloped virus budding: can some viruses do without an ESCRT? Virology 372, 221–232.
Corless, L., Crump, C.M., Griffin, S.D., Harris, M., 2010. Vps4 and the ESCRT-III complex are required for the release of infectious hepatitis C virus particles. J. Gen. Virol. 91, 362–372.
de Gassart, A., Geminard, C., Hoekstra, D., Vidal, M., 2004. Exosome secretion: the art of reutilizing nonrecycled proteins? Traffic 5, 896–903.
Denzer, K., Kleijmeer, M.J., Heijnen, H.F., Stoorvogel, W., Geuze, H.J., 2000. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. J. Cell Sci. 113 (Pt 19), 3365–3374.
Eden, E.R., White, I.J., Tsapara, A., Futter, C.E., 2010. Membrane contacts between endosomes and ER provide sites for PTP1B-epidermal growth factor receptor interaction. Nat. Cell Biol. 12, 267–272.
Evans, M.J., von Hahn, T., Tscherne, D.M., Syder, A.J., Panis, M., Wolk, B., Hatziioannou, T., McKeating, J.A., Bieniasz, P.D., Rice, C.M., 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 446, 801–805.
Gottwein, E., Krausslich, H.G., 2005. Analysis of human immunodeficiency virus type 1 Gag ubiquitination. J. Virol. 79, 9134–9144.
Gould, S.J., Booth, A.M., Hildreth, J.E., 2003. The Trojan exosome hypothesis. Proc. Natl. Acad. Sci. U.S.A. 100, 10592–10597.
Iwata, K., Yamamoto, A., Satoh, S., Ohyama, Y., Tashiro, Y., Setoguchi, T., 1995. Quantitative immunoelectron microscopic analysis of the localization and induction of 25-hydroxyvitamin D3 24-hydroxylase in rat kidney. J. Histochem. Cytochem. 43, 255–262.
Kato, T., Date, T., Murayama, A., Morikawa, K., Akazawa, D., Wakita, T., 2006. Cell culture and infection system for hepatitis C virus. Nat. Protoc. 1, 2334–2339.
Kolegraff, K., Bostik, P., Ansari, A.A., 2006. Characterization and role of lentivirus-associated host proteins. Exp. Biol. Med. (Maywood) 231, 252–263.
Komada, M., Kitamura, N., 1995. Growth factor-induced tyrosine phosphorylation of Hrs, a novel 115-kilodalton protein with a structurally conserved putative zinc finger domain. Mol. Cell. Biol. 15, 6213–6221.
Kyuuma, M., Kikuchi, K., Kojima, K., Sugawara, Y., Sato, M., Mano, N., Goto, J., Takeshita, T., Yamamoto, A., Sugamura, K., Tanaka, N., 2007. AMSH, an ESCRT-III associated enzyme, deubiquitinates cargo on MVB/late endosomes. Cell Struct. Funct. 31, 159–172.
Lai, C.K., Jeng, K.S., Machida, K., Lai, M.M., 2010. Hepatitis C virus egress and release depend on endosomal trafficking of core protein. J. Virol. 84, 11590–11598.
Lenassi, M., Cagney, G., Liao, M., Vaupotic, T., Bartholomeeusen, K., Cheng, Y., Krogan, N.J., Plemenitas, A., Peterlin, B.M., 2010. HIV Nef is secreted in exosomes and triggers apoptosis in bystander CD4+ T cells. Traffic 11, 110–122.
Masciopinto, F., Giovani, C., Campagnoli, S., Galli-Stampino, L., Colombatto, P., Brunetto, M., Yen, T.S., Houghton, M., Pileri, P., Abrignani, S., 2004. Association of hepatitis C virus envelope proteins with exosomes. Eur. J. Immunol. 34, 2834–2842.
McDonald, B., Martin-Serrano, J., 2009. No strings attached: the ESCRT machinery in viral budding and cytokinesis. J. Cell Sci. 122, 2167–2177.
Miyanari, Y., Atsuzawa, K., Usuda, N., Watashi, K., Hishiki, T., Zayas, M., Bartenschlager, R., Wakita, T., Hijikata, M., Shimotohno, K., 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nat. Cell Biol. 9, 1089–1097.
Mori, Y., Koike, M., Moriishi, E., Kawabata, A., Tang, H., Oyaizu, H., Uchiyama, Y., Yamanishi, K., 2008. Human herpesvirus-6 induces MVB formation, and virus egress occurs by an exosomal release pathway. Traffic 9, 1728–1742.
Murray, C.L., Jones, C.T., Rice, C.M., 2008. Architects of assembly: roles of Flaviviridae non-structural proteins in virion morphogenesis. Nat. Rev. Microbiol. 6, 699–708.
Ostrowski, M., Carmo, N.B., Krumeich, S., Fanget, I., Raposo, G., Savina, A., Moita, C.F., Schauer, K., Hume, A.N., Freitas, R.P., Goud, B., Benaroch, P., Hacohen, N., Fukuda, M., Desnos, C., Seabra, M.C., Darchen, F., Amigorena, S., Moita, L.F., Thery, C., 2010. Rab27a and Rab27b control different steps of the exosome secretion pathway. Nat. Cell Biol. 12, 19–30 (sup pp 11–13).
Pan, B.T., Teng, K., Wu, C., Adam, M., Johnstone, R.M., 1985. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. J. Cell Biol. 101, 942–948.
Pornillos, O., Higginson, D.S., Stray, K.M., Fisher, R.D., Garrus, J.E., Payne, M., He, G.P., Wang, H.E., Morham, S.G., Sundquist, W.I., 2003. HIV Gag mimics the Tsg101-recruiting activity of the human Hrs protein. J. Cell Biol. 162, 425–434.
Savina, A., Fader, C.M., Damiani, M.T., Colombo, M.I., 2005. Rab11 promotes docking and fusion of multivesicular bodies in a calcium-dependent manner. Traffic 6, 131–143.
Shiina, M., Rehermann, B., 2008. Cell culture-produced hepatitis C virus impairs plasmacytoid dendritic cell function. Hepatology 47, 385–395.
Shirakura, M., Murakami, K., Ichimura, T., Suzuki, R., Shimoji, T., Fukuda, K., Abe, K., Sato, S., Fukasawa, M., Yamakawa, Y., Nishijima, M., Moriishi, K., Matsuura, Y., Wakita, T., Suzuki, T., Howley, P.M., Miyamura, T., Shoji, I., 2007. E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. J. Virol. 81, 1174–1185.
Takeuchi, T., Katsume, A., Tanaka, T., Abe, A., Inoue, K., Tsukiyama-Kohara, K., Kawaguchi, R., Tanaka, S., Kohara, M., 1999. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 116, 636–642.
Tamai, K., Tanaka, N., Nara, A., Yamamoto, A., Nakagawa, I., Yoshimori, T., Ueno, Y., Shimosegawa, T., Sugamura, K., 2007. Role of Hrs in maturation of autophagosomes in mammalian cells. Biochem. Biophys. Res. Commun. 360, 721–727.
Tamai, K., Tanaka, N., Nakano, T., Kakazu, E., Kondo, Y., Inoue, J., Shiina, M., Fukushima, K., Hoshino, T., Sano, K., Ueno, Y., Shimosegawa, T., Sugamura, K., 2010. Exosome

secretion of dendritic cells is regulated by Hrs, an ESCRT-0 protein. Biochem. Biophys. Res. Commun. 399, 384–390.
Tanaka, Y., Tanaka, N., Saeki, Y., Tanaka, K., Murakami, M., Hirano, T., Ishii, N., Sugamura, K., 2008. c-Cbl-dependent monoubiquitination and lysosomal degradation of gp130. Mol. Cell. Biol. 28, 4805–4818.
Tanida, I., Fukasawa, M., Ueno, T., Kominami, E., Wakita, T., Hanada, K., 2009. Knockdown of autophagy-related gene decreases the production of infectious hepatitis C virus particles. Autophagy 5, 937–945.
van Niel, G., Porto-Carreiro, I., Simoes, S., Raposo, G., 2006. Exosomes: a common pathway for a specialized function. J. Biochem. 140, 13–21.
Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11, 791–796.
Wollert, T., Hurley, J.H., 2010. Molecular mechanism of multivesicular body biogenesis by ESCRT complexes. Nature 464, 864–869.