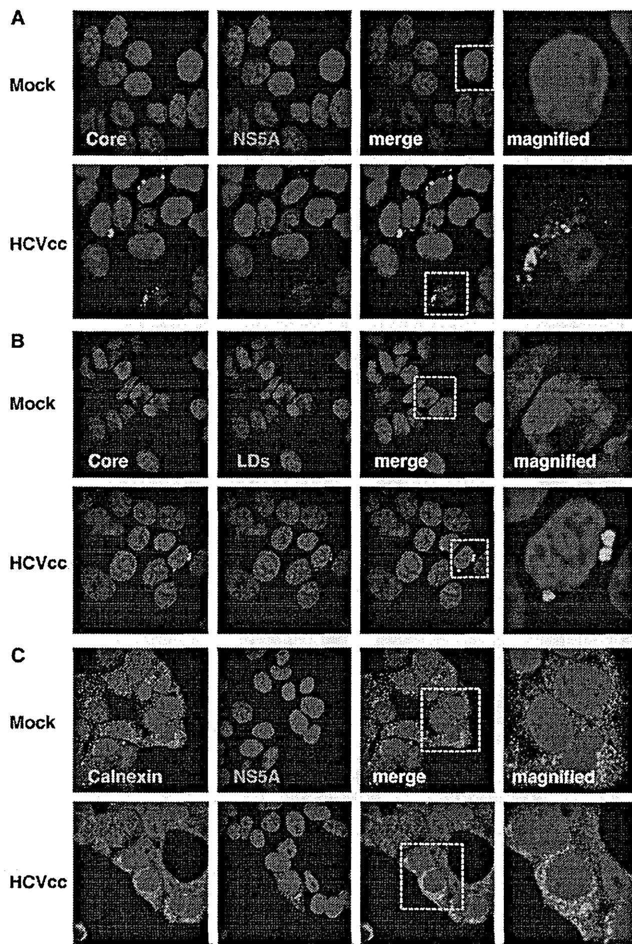


FIG 7 Subcellular localization of core and NS5A proteins in Hec1B/miR-122 cells infected with HCVcc. Hec1B/miR-122 cells infected with or without HCVcc at an MOI of 1 were fixed with 4% PFA at 48 h postinfection and stained with appropriate antibodies to core and NS5A proteins (A), core protein and lipid droplets (B), and NS5A and calnexin (C). The boxes in the merged images were magnified, and the images are displayed on the right.

MTTP, in nonhepatic cells were significantly lower than those in hepatic cells (Fig. 10). Collectively, these results suggest that intracellular functional lipid metabolism, including the biosynthesis of lipid droplets and the production of VLDL, participates in the assembly of HCV.

**Establishment of HCV replicon in Hec1B/miR-122 cells.** It was previously shown by using RNA replicon cells based on the JFH1 strain that expression of miR-122 enhanced the translation of HCV RNA in HEK293 cells and MEFs (8, 35). We tried to establish HCV replicon cells based on genotype 1b Con1 and genotype 2a JFH1 strains in Hec1B/miR-122 and HEK293 cells stably expressing miR-122 (HEK293/miR-122). To examine the colony formation efficiency of the HCV RNAs of the Con1 and JFH1 strains, SGR RNA was electroporated into the cell lines and selected by G418 for 3 weeks. Expression of miR-122 in Hec1B cells significantly enhanced the colony formation of SGR of the Con1 strain (Fig. 11A), suggesting that the expression of miR-122 in Hec1B cells supports the efficient replication of SGR. HCV replication in 20 replicon clones established by the transfection with SGR RNA of the Con1 strain in Hec1B/miR-122 cells was examined by qRT-PCR and immunoblotting. All clones contained high levels of HCV RNA ($3 \times 10^6$ to $5 \times 10^7$ copies per μg of total RNA) (Fig. 11B), and expression of NS5A was well correlated with the levels of HCV RNA in the clones (Fig. 11C). Two replicon clones (clones 2 and 10) in Hec1B/miR-122 cells exhibiting high levels of RNA replication and NS5A expression further confirmed the high level of expression of NS5A by immunofluorescent microscopy (Fig. 11D). These results suggest that expression of miR-122 facilitates the efficient replication of SGR of at least two HCV genotypes in Hec1B cells.

Our previous reports showed that HCV NS proteins were colocalized with dsRNA and cochaperone molecules, FK506-binding protein 8 (FKBP8), in dot-like structures on the ER membrane of Huh7 replicon cells (59). Colocalization of NS5A with dsRNA or FKBP8 was observed in the dot-like structures in not only Huh7 SGR cells but also Hec1B/miR-122 SGR cells (Fig. 12A), suggesting that the dot-like structure required for efficient viral replication is also generated in Hec1B/miR-122 replicon cells. It has been shown that HCV replication induces the formation of convoluted membranous structures, called membranous webs, in Huh7 cells (13, 45). FM-EM techniques revealed the localization of NS5A on the convoluted structures in Hec1B/miR-122 replicon cells (Fig. 12B). These results suggest that the replication complex required for viral replication was also generated in the Hec1B/miR-122 replicon cells, as was seen in the Huh7 replicon cells.

**miR-122 is a crucial determinant of HCVcc propagation.** It has been shown that the infectivity of HCVcc in cured cells, established when IFN treatment induces the elimination of the viral genome from the Huh7 replicon cells harboring an HCV RNA, is significantly higher than that in parental Huh7 cells (2, 66). Therefore, we tried to establish Hec1B-based cured cells from the Con1 SGR clones harboring a high copy number of HCV RNA. Treatment with cyclosporine and the protease inhibitor of HCV suppressed NS5A expression in Hec1B/miR-122 SGR clone 2 in a dose-dependent manner (Fig. 13A), whereas no reduction was observed by the IFN treatment due to a lack of an IFN receptor, as shown in Fig. 4C. It was reported that monotherapy by the HCV protease inhibitor induces the emergence of resistant breakthrough viruses (34, 55). Therefore, we treated five Hec1B/miR-122 SGR clones (clones 2, 5, 10, 14, and 16) with 1 μg/ml cyclosporine and 100 nM protease inhibitor for HCV. Viral RNA was determined by qRT-PCR every 5 days posttreatment. Elimination of viral RNA was achieved in four clones (clones 2, 5, 10, and 14) within 20 days posttreatment (Fig. 13B). Replication of HCV RNA in the cured cells infected with HCVcc at an MOI of 0.5 was 2- to 30-fold higher than that in parental cells at 24 h postinfection (Fig. 13C). In addition, replication of HCV RNA in cured clone 2 infected with HCVcc at an MOI of 0.1 was comparable to that in Huh7.5.1 cells until 24 h postinfection (Fig. 13D). Expression of NS5A was significantly increased in cured clone 2 compared to that in the parental Hec1B/miR-122 cells (Fig. 13E and F). It was previously shown that the increased permissiveness of Huh7-derived cured cells, Huh7.5 cells, is attributable to a mutation in the RIG-I gene (58). To examine the innate immune response in the parental and cured Hec1B/miR-122 cells, induction of IFN-stimulated gene 15 (ISG15) was determined upon stimulation with IFN-α or VSV. Although induction of ISG15 was not observed in either parental or cured cells upon stimulation with IFN-α due to a lack of an IFN receptor (11) (Fig. 14A), it was

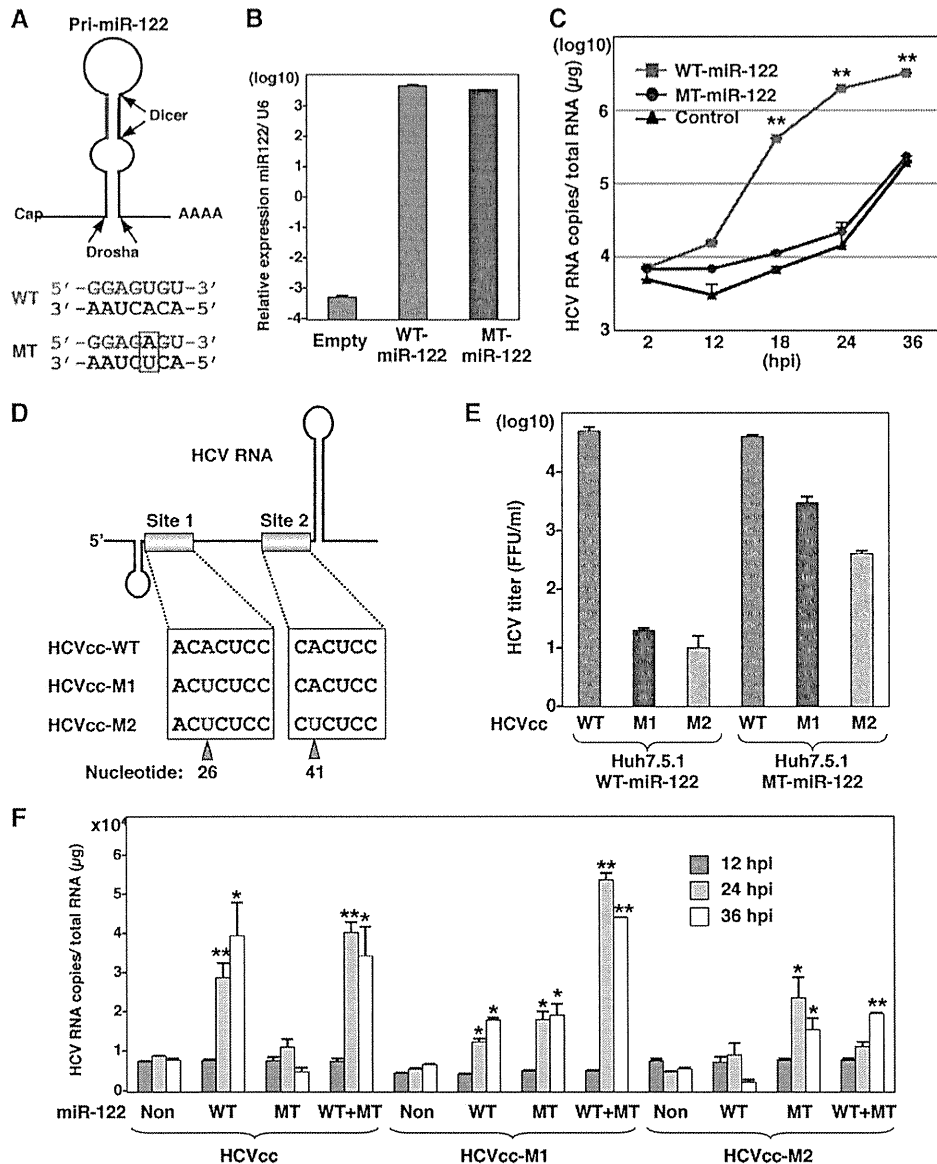


FIG 8 Specific interaction between miR-122 and the 5′ UTR of HCV is required for HCV replication. (A) Structures of pri-miR-122 and the nucleotide sequences of WT and MT miR-122, which has a substitution of uridine to adenosine in the seed domain and an additional complementary substitution of adenosine to uridine for stable expression. (B) WT or MT miR-122 was introduced into Hec1B cells by a lentiviral vector, and miR-122 expression levels were determined by qRT-PCR. (C) HCVcc was inoculated into Hec1B cells expressing either WT or MT miR-122 and control cells at an MOI of 1, and the intracellular HCV RNA levels were determined by qRT-PCR. (D) Diagram of mutant viruses HCVcc-M1 and HCVcc-M2 carrying complementary substitutions in the miR-122-binding site 1 alone and both sites 1 and 2 in the 5′ UTR of HCV, respectively. (E) Viral RNA of HCVcc, HCVcc-M1, or HCVcc-M2 was electroporated into Huh7.5.1 cells expressing either WT or MT miR-122, and infectious titers of the viruses recovered in the culture supernatants at 72 h postinfection of the second passage were determined by a focus-forming assay in cells expressing either WT or MT miR-122. Red, blue, and green bars, infectious titers of HCVcc, HCVcc-M1, and HCV-M2, respectively. (F) HCVcc, HCVcc-M1, or HCV-M2 was inoculated into Hec1B cells expressing either or both WT and MT miR-122 at an MOI of 0.5, and intracellular HCV RNA levels were determined at 12, 24, and 36 h postinfection by qRT-PCR. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for control cells.

detected in both cells infected with VSV (Fig. 14B). Therefore, other mechanisms should be involved in the enhancement of permissiveness of Hec1B-derived cured cells. Ehrhardt et al. showed that the expression levels of miR-122 in Huh7-derived cured cells, including Huh7.5, Huh7.5.1, and Huh7-Lunet cells, are significantly higher than those in parental Huh7 cells (14). In addition, our recent study indicated that levels of expression of miR-122 in the cured Huh7 and Hep3B/miR-122 cells were higher than those in parental cells (29). Levels of expression of miR-122 in the Hec1B-based cured cell clones are also higher than those in parental Hec1B/miR-122 cells (Fig. 13G). These results suggest that a high level of miR-122 expression is a crucial determinant of high susceptibility to HCVcc propagation in the cured cells.

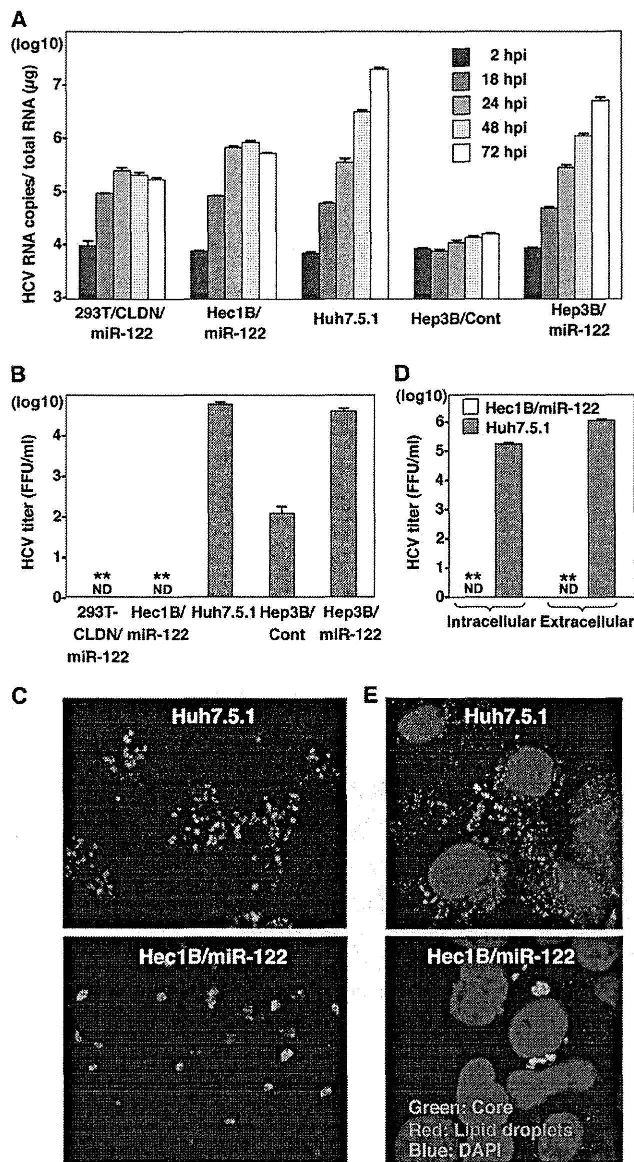


FIG 9 Viral particle formation in hepatic and nonhepatic cells. (A) HCVcc was inoculated into 293T-CLDN/miR-122, Hec1B/miR-122, Hep3B/Cont, and Hep3B/miR-122 cells at an MOI of 1 and into Huh7.5.1 cells at an MOI of 0.1. HCV RNA levels (copies/μg) in cells at 2, 18, 24, 48, and 72 h postinfection were determined by qRT-PCR. (B) HCVcc was inoculated into 293T-CLDN/miR-122, Hec1B/miR-122, Hep3B/Cont, and Hep3B/miR-122 cells at an MOI of 1 or into Huh7.5.1 cells at an MOI of 0.1, and infectious titers in the culture supernatants were determined at 72 h postinfection by a focus-forming assay in Huh7.5.1 cells. ND, not determined. (C) Huh7.5.1 and Hec1B/miR-122 cells were infected with HCVcc at MOIs of 0.1 and 1, respectively, incubated with 1% methylcellulose in DMEM containing 10% FCS for 72 h, fixed with 4% PFA, and subjected to immunofluorescence analysis using anti-NS5A antibody. (D) Hec1B/miR-122 and Huh7.5.1 cells were infected with HCVcc at MOIs of 1 and 0.1, respectively, and infectious titers in cells and supernatants were determined by a focus-forming assay at 72 h postinfection. (E) Huh7.5.1 and Hec1B/miR-122 cells were infected with HCVcc at MOIs of 0.1 and 1, respectively, fixed with 4% PFA, and subjected to immunofluorescence assay using anti-core protein antibody (green). Lipid droplets and cell nuclei were stained with BODIPY (red) and DAPI (blue), respectively. Asterisks indicate significant differences (**, $P < 0.01$) versus the results for Huh7.5.1 cells.

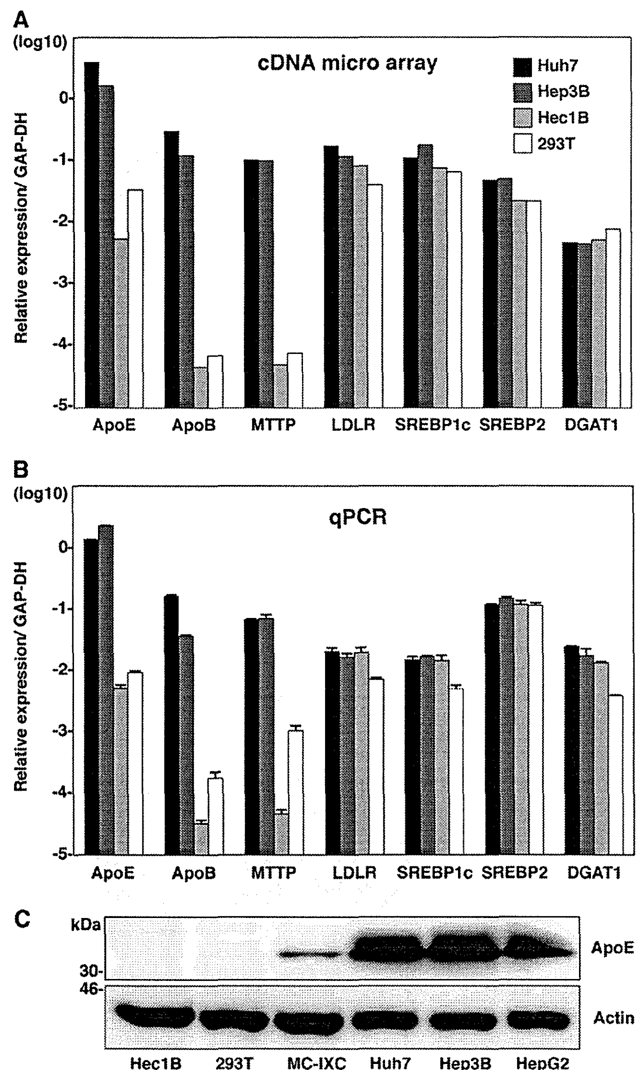


FIG 10 Expression of lipid metabolism-associated proteins in hepatic and nonhepatic cells. (A) Expression levels of ApoE, ApoB, MTTP, LDLR, SREBP1c, SREBP2, and DGAT1 were compared among hepatic (Huh7 and Hep3B) and nonhepatic (Hec1B and 293T) cells using cDNA microarray analyses. (B) Total RNA was extracted from the cells, and expression levels of ApoE, ApoB, MTTP, LDLR, SREBP1c, SREBP2, and DGAT1 gene were determined by qPCR. (C) Nonhepatic (Hec1B, 293T, and MC-IXC) and hepatic (Huh7, Hep3B, and HepG2) cells were subjected to immunoblotting using anti-ApoE antibody.

## DISCUSSION

Although multiple epidemiological studies have revealed that HCV infection induces several EHMs, they have not well elucidated the molecular mechanisms of the EHMs induced by HCV infection (19). Indeed, HCVcc does not infect PBMCs (38). It has been shown that two neuroepithelioma cell lines permit HCVcc infection at low levels (17) and lymphotropic strains or quasispecies of HCV exist in infected individuals (12, 52). Furthermore, many molecules involved in the entry, replication, and assembly of HCVcc have been identified, although these molecules are not sufficient to explain the liver tropism of HCV. Recently, a liver-specific microRNA, miR-122, was shown to facilitate the efficient

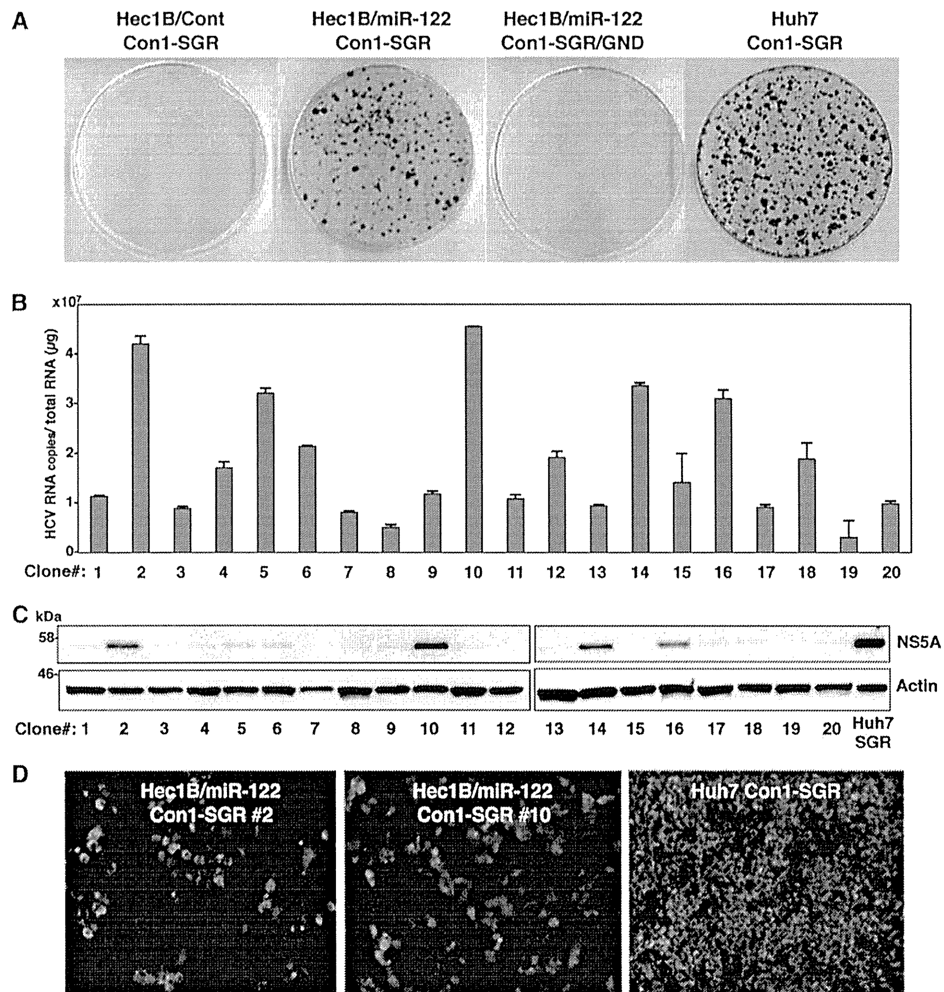


FIG 11 Establishment of Con1-based HCV replicon cells by using Hec1B cells. (A) WT or replication-defective SGR RNA of the HCV Con1 strain was electroporated into Hec1B/Cont, Hec1B/miR-122, and Huh7 cells, and the medium was replaced with DMEM containing 10% FCS and 1 mg/ml G418 at 24 h postelectroporation. Colonies were stained with crystal violet after 3 weeks of selection with G418. (B) Total RNAs of 20 selected clones were extracted and subjected to qRT-PCR. (C) The 20 SGR clones were subjected to immunoblotting using anti-NS5A antibody. Huh7-derived Con1-based SGR cells were used as a positive control. (D) NS5A proteins in SGR clones 2 and 10 were stained with appropriate antibodies and examined by fluorescence microscopy. Huh7-derived Con1-based SGR and parental Hec1B cells were used for positive and negative controls, respectively.

replication of HCV through a specific interaction with the complementary sequences in the 5′ UTR of HCV RNA (21, 25, 27, 36). In addition, exogenous expression of miR-122 facilitates the replication of SGR of the JFH1 strain in HEK293 cells (8) and the propagation of HCVcc in HepG2 and Hep3B nonpermissive hepatoma cells (29, 43), suggesting that the expression of miR-122 is required for the efficient replication of HCV. However, HCV replicon cells have also been established in HeLa and LI90 cells derived from stellate cells in which no exogenous miR-122 is expressed (30, 63). In this study, naïve Hec1B cells also exhibited a low level of replication upon infection with HCVcc (Fig. 4B), and this replication was resistant to treatment with an inhibitor of miR-122, LNA-miR-122 (Fig. 6C), suggesting that miR-122 expression is not a necessary condition but is required for the enhancement of HCV replication and that HCV is capable of replicating in nonhepatic cells in an miR-122-independent manner. Although the application of miR-122-specific LNAs to chronic hepatitis C patients is now in progress (32), further studies are needed to clarify the mechanisms underlying the miR-122-independent replication of HCV in more detail.

Although the importance of receptor-mediated entry in the cell tropism of HCV has been evaluated (16, 65), cDNA microarray databases, including the NextBio search engine, revealed that HCV receptor candidates, including hCD81, SR-BI, CLDN1, and OCLN, are highly expressed in many nonhepatic tissues. In addition, our current data and previous reports demonstrated that many nonhepatic cells permitted the entry of the pseudotype viruses bearing HCV envelope proteins, suggesting that other host factors must be involved in the cell tropism of HCV to human hepatocytes (4, 17, 54, 61). The data in this study suggest that miR-122 expression and functional lipid metabolism play crucial roles in the determination of an efficient propagation of HCV *in vitro*. On the other hand, previous studies showed the compartmentalization of genetic variation in HCV between hepatic and

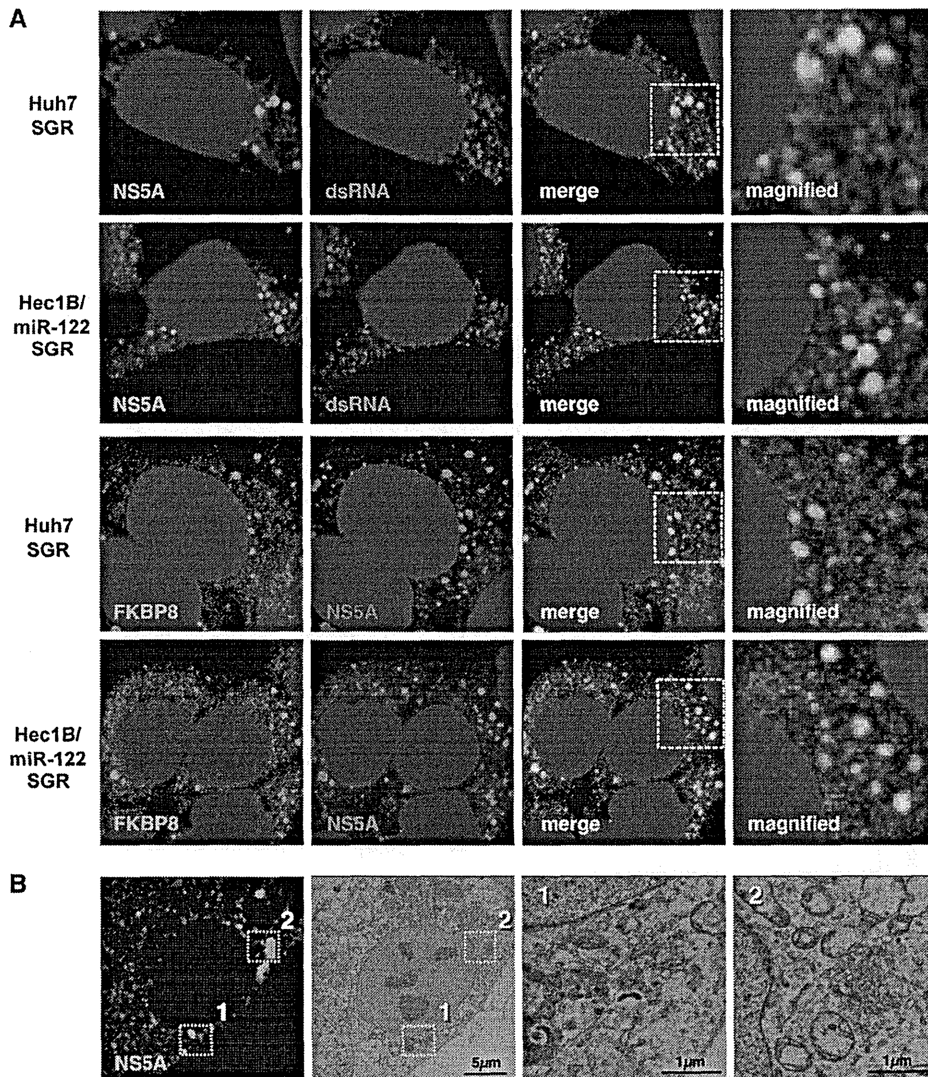


FIG 12 Replication complex in Hec1B/miR-122 replicon cells. (A) Huh7 and Hec1B/miR-122 cells harboring the Con1 SGR RNA were fixed, permeabilized, and stained with antibodies to NS5A and dsRNA or FKBP8. The boxed areas in the merged images were magnified, and the images are displayed on the right. (B) Hec1B-derived Con1 SGR cells were stained with anti-NS5A antibody. Identical fields were observed under EM by using the correlative FM-EM technique. The boxed areas are magnified, and the images displayed on the right.

nonhepatic tissues, suggesting that HCV is capable of replicating in nonhepatic tissues expressing either miR-122, ApoE, ApoB, or MTTP (52). Collectively, these results suggest that entry receptors, miR-122, and functional lipid metabolism are mainly involved in the regulation of internalization, RNA replication, and assembly of HCV, respectively, and are important factors in determining the cell tropism of HCV to hepatocytes. On the other hand, it might be feasible to speculate that EHMs observed in chronic hepatitis C patients are caused by an incomplete miR-122-independent propagation of HCV in nonhepatic cells.

In spite of the efficient replication of HCV in Hec1B and 293T-CLDN cells expressing miR-122, no infectious particle was detected, in contrast to the case with hepatic cells (Fig. 9), suggesting the involvement of liver-specific host factors and/or machineries in the assembly of infectious particles. In general, the liver plays a major role in lipid metabolism, such as in fatty acid and lipoprotein syntheses (9), and many reports have indicated the involvement of lipid metabolism, especially triglyceride metabolism, in the assembly and budding of HCV particles. Lipid droplets, MTTP, ApoB, and ApoE have been shown to participate in the assembly and secretion of infectious particles of HCVcc in Huh7 cells (20, 23, 26, 40). In the current analyses, there were fewer lipid droplets and the expression levels of ApoE, ApoB, and MTTP were lower in nonhepatic cells than in hepatic cells. Although minus-strand HCV RNA and viral proteins were detected in nonhepatic cells (33, 60), it was shown that the recurrence of HCV after liver transplantation for patients with HCV-induced liver diseases was mainly caused by HCV variants generated in the liver but not in nonhepatic tissues (50). These results support the notion that replication of HCV RNA in nonhepatic cells is unlikely to be a reservoir for persistent infection, due to the lack of infectious particle formation.

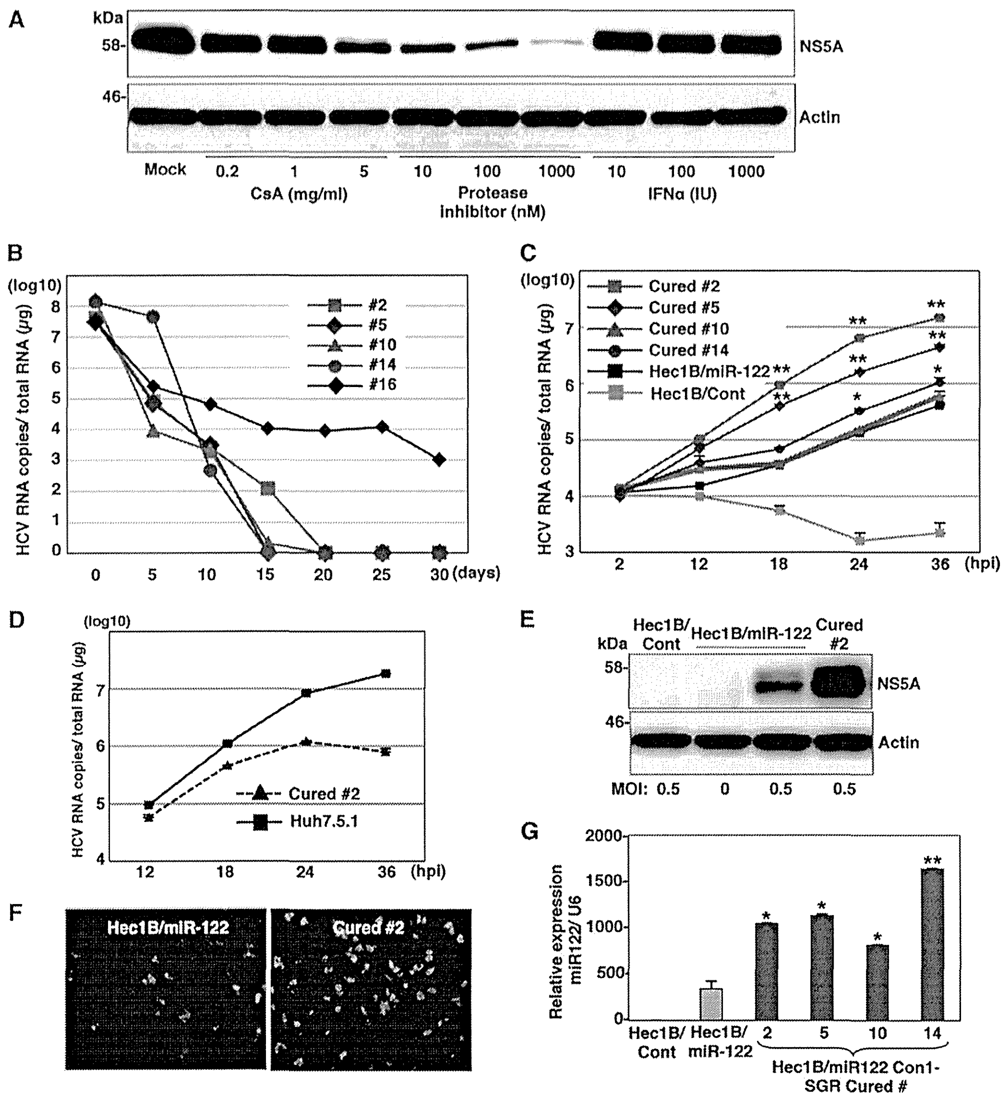


FIG 13 miR-122 is a crucial determinant for the efficient replication of HCVcc and replicon RNA. (A) Hec1B Con1 replicon clone 2 was treated with stepwise concentrations of cyclosporine (CsA; 0.2, 1, and 5 μg/ml), NS3/4A protease inhibitor (10, 100, and 1,000 nM), or IFN-α (10, 100, and 1,000 IU) and subjected to immunoblotting using anti-NS5A antibody at 48 h posttreatment. (B) Five Con1-based SGR clones were treated with the combination of 1 μg/ml cyclosporine and 100 nM HCV protease inhibitor to eliminate the HCV genome. Intracellular HCV RNA levels at 5, 10, 15, 20, 25, and 30 days posttreatment were determined by qRT-PCR analysis. (C) HCVcc was inoculated with Hec1B/Cont, parental Hec1B/miR-122, and Hec1B-based cured cells (clones 2, 5, 10, and 14) at an MOI of 1. Intracellular HCV RNA levels at 2, 12, 18, 24, and 36 h postinfection were determined by qRT-PCR analysis. (D) HCVcc was inoculated into Huh7.5.1 and Hec1B/miR-122 cured clone 2 cells at an MOI of 1. Intracellular HCV RNA levels were determined by qRT-PCR at 12, 24, 36, and 48 h postinfection. (E and F) Hec1B/Cont, parental Hec1B/miR-122, and cured cells of clone 2 were infected with HCVcc at an MOI of 0.5. After 48 h, the cells were subjected to immunoblotting and immunofluorescence analyses using appropriate antibodies. (G) Total miRNAs were extracted from Hec1B/Cont (white), parental Hec1B-miR-122 cells (gray), and four cured cell clones (black). miR-122 expression levels in these cells were determined by qRT-PCR analysis. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) versus the results for parental Hec1B/miR-122 cells.

The endogenous expression of miR-122 is hardly detected in Hec1B cells, in contrast to the abundant expression of miR-122 in Huh7 cells. Therefore, more accurate analyses of the biological significance of the interaction between miR-122 and the 5′ UTR on the replication of HCVcc in Hec1B could be possible by introducing mutations not only into viruses but also into miR-122. Replication of HCVcc and a mutant virus bearing two mutations in the 5′ UTR (HCVcc-M2) was observed in Hec1B cells expressing WT and MT miR-122, respectively, although the level of replication was lower in cells infected with HCVcc-M2 than in those infected with HCVcc, probably due to the mutations in the 5′ UTR (Fig. 8F). In contrast, a mutant virus (HCVcc-M1) bearing a mutation in site 1 alone exhibited efficient replication in Hec1B cells expressing both WT and MT miR-122 comparable to the replication level of the wild-type virus in cells expressing WT miR-122. Furthermore, the replication level of HCVcc-M1 was low in Hec1B cells expressing either WT or MT miR-122, suggesting that interaction between miR-122 and either of the seed sequence-binding sites in the 5′ UTR has an equal ability to enhance the replication of the HCV genome. However, it was shown that the

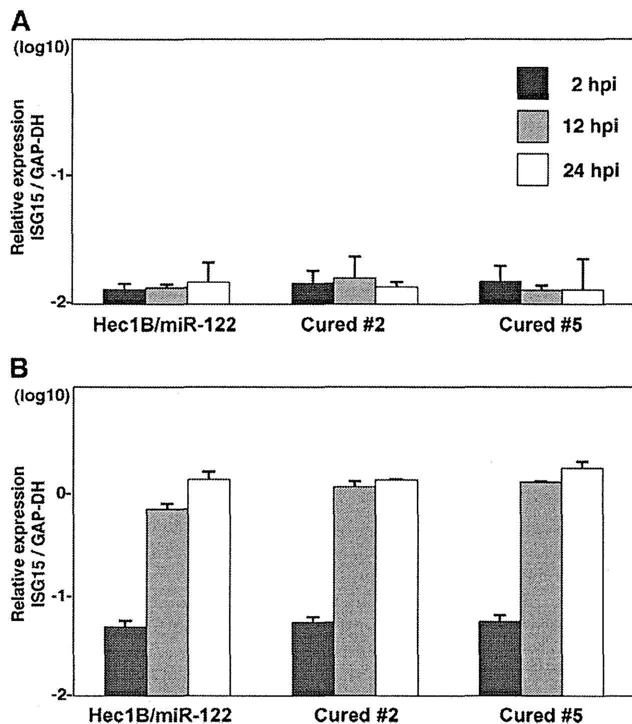


FIG 14 Innate immune responses in parental and cured Hec1B/miR-122 cells. Parental and cured Hec1B/miR-122 cells were stimulated with 100 U of IFN-α (A) or VSV (B). The expression levels of IFN-stimulated gene 15 (ISG15) were determined by qRT-PCR at 2, 12, and 24 h posttreatment.

ability of miR-122 to promote the growth of a laboratory strain of HCV (HJ3-5) is dependent upon its direct interaction with both seed sequence-binding sites in the 5′ UTR and that the binding to site 1 is more important for efficient replication than the binding to site 2 (25). Recently, it was shown that the binding of miR-122 to the 5′ UTR of the HCV genome masks the 5′-terminal sequences of the viral genome through the 3′ overhanging nucleotides of miR-122 (36). It is necessary to evaluate the importance of this enhancement mechanism on mutant HCVcc infection in Hec1B cells.

In summary, we demonstrated that HCV is capable of replicating at a low level in nonhepatic cells and that exogenous expression of miR-122 facilitates efficient viral replication but not the production of infectious particles, probably due to the lack of hepatocytic lipid metabolism in nonhepatic cell lines. These results suggest that miR-122 plays a crucial role in determination of the cell tropism of HCV and the possible involvement of incomplete propagation of HCV in the development of EHM in hepatitis C patients.

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## REFERENCES

1. Bartel DP. 2009. MicroRNAs: target recognition and regulatory functions. Cell 136:215–233.
2. Blight KJ, McKeating JA, Rice CM. 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. 76: 13001–13014.
3. Bukh J. 2004. A critical role for the chimpanzee model in the study of hepatitis C. Hepatology 39:1469–1475.
4. Burgel B, et al. 2011. Hepatitis C virus enters human peripheral neuroblastoma cells—evidence for extra-hepatic cells sustaining hepatitis C virus penetration. J. Viral Hepat. 18:562–570.
5. Burns DM, D'Ambrogio A, Nottrott S, Richter JD. 2011. CPEB and two poly(A) polymerases control miR-122 stability and p53 mRNA translation. Nature 473:105–108.
6. Calleja JL, et al. 1999. Sustained response to interferon-alpha or to interferon-alpha plus ribavirin in hepatitis C virus-associated symptomatic mixed cryoglobulinaemia. Aliment. Pharmacol. Ther. 13:1179–1186.
7. Castoldi M, et al. 2011. The liver-specific microRNA miR-122 controls systemic iron homeostasis in mice. J. Clin. Invest. 121:1386–1396.
8. Chang J, et al. 2008. Liver-specific microRNA miR-122 enhances the replication of hepatitis C virus in nonhepatic cells. J. Virol. 82:8215–8223.
9. Coppack SW, Jensen MD, Miles JM. 1994. In vivo regulation of lipolysis in humans. J. Lipid Res. 35:177–193.
10. Cun W, Jiang J, Luo G. 2010. The C-terminal alpha-helix domain of apolipoprotein E is required for interaction with nonstructural protein 5A and assembly of hepatitis C virus. J. Virol. 84:11532–11541.
11. Daly C, Reich NC. 1993. Double-stranded RNA activates novel factors that bind to the interferon-stimulated response element. Mol. Cell. Biol. 13:3756–3764.
12. Di Liberto G, et al. 2006. Clinical and therapeutic implications of hepatitis C virus compartmentalization. Gastroenterology 131:76–84.
13. Egger D, et al. 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J. Virol. 76:5974–5984.
14. Ehrhardt M, et al. 2011. Profound differences of microRNA expression patterns in hepatocytes and hepatoma cell lines commonly used in hepatitis C virus studies. Hepatology 54:1112–1113.
15. Elmen J, et al. 2008. LNA-mediated microRNA silencing in non-human primates. Nature 452:896–899.
16. Evans MJ, et al. 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 446:801–805.
17. Fletcher NF, et al. 2010. Hepatitis C virus infection of neuroepithelioma cell lines. Gastroenterology 139:1365–1374.
18. Fukuhara T, et al. 2011. Intracellular delivery of serum-derived hepatitis C virus. Microbes Infect. 13:405–412.
19. Galossi A, Guarisco R, Bellis L, Puoti C. 2007. Extrahepatic manifestations of chronic HCV infection. J. Gastrointestin. Liver Dis. 16:65–73.
20. Gastaminza P, et al. 2008. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. J. Virol. 82:2120–2129.
21. Henke JI, et al. 2008. microRNA-122 stimulates translation of hepatitis C virus RNA. EMBO J. 27:3300–3310.
22. Herker E, et al. 2010. Efficient hepatitis C virus particle formation requires diacylglycerol acyltransferase-1. Nat. Med. 16:1295–1298.
23. Hishiki T, et al. 2010. Infectivity of hepatitis C virus is influenced by association with apolipoprotein E isoforms. J. Virol. 84:12048–12057.
24. Huntzinger E, Izaurralde E. 2011. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat. Rev. Genet. 12:99–110.
25. Jangra RK, Yi M, Lemon SM. 2010. Regulation of hepatitis C virus translation and infectious virus production by the microRNA miR-122. J. Virol. 84:6615–6625.
26. Jiang J, Luo G. 2009. Apolipoprotein E but not B is required for the formation of infectious hepatitis C virus particles. J. Virol. 83:12680–12691.
27. Jopling CL, Schutz S, Sarnow P. 2008. Position-dependent function for a tandem microRNA miR-122-binding site located in the hepatitis C virus RNA genome. Cell Host Microbe 4:77–85.
28. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. 2005. Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. Science 309:1577–1581.
29. Kambara H, et al. 2012. Establishment of a novel permissive cell line for

propagation of hepatitis C virus by the expression of microRNA miR122. J. Virol. 86:1382–1393.
30. Kato T, et al. 2005. Nonhepatic cell lines HeLa and 293 support efficient replication of the hepatitis C virus genotype 2a subgenomic replicon. J. Virol. 79:592–596.
31. Kupershmidt IQ, et al. 2010. Ontology-based meta-analysis of global collections of high-throughput public data. PLoS One 5:e13066. doi: 10.1371/journal.pone.0013066.
32. Lanford RE, et al. 2010. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 327:198–201.
33. Lerat H, et al. 1996. Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. J. Clin. Invest. 97:845–851.
34. Lin C, et al. 2004. In vitro resistance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061: structural analysis indicates different resistance mechanisms. J. Biol. Chem. 279:17508–17514.
35. Lin LT, et al. 2010. Replication of subgenomic hepatitis C virus replicons in mouse fibroblasts is facilitated by deletion of interferon regulatory factor 3 and expression of liver-specific microRNA 122. J. Virol. 84:9170–9180.
36. Machlin ES, Sarnow P, Sagan SM. 2011. Masking the 5′ terminal nucleotides of the hepatitis C virus genome by an unconventional microRNA-target RNA complex. Proc. Natl. Acad. Sci. U. S. A. 108:3193–3198.
37. Manns MP, et al. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 358:958–965.
38. Marukian S, et al. 2008. Cell culture-produced hepatitis C virus does not infect peripheral blood mononuclear cells. Hepatology 48:1843–1850.
39. Masaki T, et al. 2010. Production of infectious hepatitis C virus by using RNA polymerase I-mediated transcription. J. Virol. 84:5824–5835.
40. Miyanari Y, et al. 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nat. Cell Biol. 9:1089–1097.
41. Moriishi K, et al. 2010. Involvement of PA28gamma in the propagation of hepatitis C virus. Hepatology 52:411–420.
42. Morris T, Robertson B, Gallagher M. 1996. Rapid reverse transcription-PCR detection of hepatitis C virus RNA in serum by using the TaqMan fluorogenic detection system. J. Clin. Microbiol. 34:2933–2936.
43. Narbus CM, et al. 2011. HepG2 cells expressing microRNA miR-122 support the entire hepatitis C virus life cycle. J. Virol. 85:12087–12092.
44. Okamoto T, et al. 2006. Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. EMBO J. 25:5015–5025.
45. Okamoto T, et al. 2008. A single-amino-acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication. J. Virol. 82:3480–3489.
46. Pawlotsky JM, Chevaliez S, McHutchison JG. 2007. The hepatitis C virus life cycle as a target for new antiviral therapies. Gastroenterology 132: 1979–1998.
47. Pietschmann T, et al. 2002. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J. Virol. 76:4008–4021.
48. Pileri P, et al. 1998. Binding of hepatitis C virus to CD81. Science 282: 938–941.
49. Ploss A, et al. 2009. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature 457:882–886.
50. Ramirez S, et al. 2009. Hepatitis C virus compartmentalization and infection recurrence after liver transplantation. Am. J. Transplant. 9:1591–1601.
51. Rieder CL, Bowser SS. 1985. Correlative immunofluorescence and electron microscopy on the same section of Epon-embedded material. J. Histochem. Cytochem. 33:165–171.
52. Roque-Afonso AM, et al. 2005. Compartmentalization of hepatitis C virus genotypes between plasma and peripheral blood mononuclear cells. J. Virol. 79:6349–6357.
53. Russell RS, et al. 2008. Advantages of a single-cycle production assay to study cell culture-adaptive mutations of hepatitis C virus. Proc. Natl. Acad. Sci. U. S. A. 105:4370–4375.
54. Sainz B, Jr, et al. 2012. Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. Nat. Med. 18:281–285.
55. Sarrazin C, et al. 2007. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. Gastroenterology 132:1767–1777.
56. Scarselli E, et al. 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. 21:5017–5025.
57. Seeff LB. 2002. Natural history of chronic hepatitis C. Hepatology 36:S35–S46. doi:10.1002/hep.1840360706.
58. Sumpter RJ, et al. 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J. Virol. 79:2689–2699.
59. Taguwa S, et al. 2009. Cochaperone activity of human butyrate-induced transcript 1 facilitates hepatitis C virus replication through an Hsp90-dependent pathway. J. Virol. 83:10427–10436.
60. Takyar ST, Li D, Wang Y, Trowbridge R, Gowans EJ. 2000. Specific detection of minus-strand hepatitis C virus RNA by reverse-transcription polymerase chain reaction on polyA(+)-purified RNA. Hepatology 32: 382–387.
61. Tani H, et al. 2007. Replication-competent recombinant vesicular stomatitis virus encoding hepatitis C virus envelope proteins. J. Virol. 81: 8601–8612.
62. Wakita T, et al. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11:791–796.
63. Watanabe N, et al. 2011. Hepatitis C virus RNA replication in human stellate cells regulates gene expression of extracellular matrix-related molecules. Biochem. Biophys. Res. Commun. 407:135–140.
64. Wilkinson J, Radkowski M, Laskus T. 2009. Hepatitis C virus neuroinvasion: identification of infected cells. J. Virol. 83:1312–1319.
65. Yang W, et al. 2008. Correlation of the tight junction-like distribution of claudin-1 to the cellular tropism of hepatitis C virus. J. Biol. Chem. 283: 8643–8653.
66. Zhong J, et al. 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. U. S. A. 102:9294–9299.

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***Review Article***

# Membrane recruitment of autophagy proteins in selective autophagy

Eiji Morita[1,2] and Tamotsu Yoshimori[1]

[1]*Laboratory of Intracellular Membrane Dynamics, Graduate School of Frontier Biosciences and* [2]*Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*

Autophagy is a stress response that is upregulated in response to signals such as starvation, growth factor deprivation, endoplasmic reticulum stress, and pathogen infection. Defects in this pathway are the underlying cause of a number of diseases, including metabolic aberrations, infectious diseases, and cancer, which are closely related to hepatic disorders. To date, more than 30 human ATG (autophagy) genes have been reported to regulate autophagosome formation. In this review, we summarize the current understanding of how ATG proteins behave during autophagosome formation in both non-selective and selective autophagy.

**Key words:** autophagy, autophagy proteins, LC3 recruitment, salmonella autophagy

## INTRODUCTION

AUTOPHAGY IS A process of self-degradation of cellular components in which double-membrane autophagosomes sequester organelles or portions of cytosol and fuse with lysosomes so that the contents can be digested by hydrolases.[1,2] By degradation of cellular components, autophagy supplies energy so that cells can survive under starvation conditions, or regulates the balance between biogenesis and degradation of cellular structures. Autophagy also plays a role in the elimination of unwanted substances from the cytoplasm, including invading bacteria or viruses, or misfolded or aggregated proteins. Therefore, autophagy plays critical roles in growth, survival, and differentiation in eukaryotes, and defects in autophagy are associated with numerous human diseases.[1,2]

There are two different autophagic pathways, a starvation-induced non-selective pathway and a target-specific selective pathway.[2] The non-selective pathway is thought to play a role in supplying energy by degrading proteins, and the selective pathway may function to eliminate unwanted substrates from the cytoplasm. In mammalian cells, the membrane dynamics of autophagosome formation in both pathways are thought to be driven by common ATG (autophagy) protein factors that were originally identified by yeast genetic analyses.[2] The molecular mechanisms of ATG-mediated autophagosome formation have been analyzed using the yeast system, and it has generally been assumed that the mammalian ATG proteins function similarly to their counterparts in the yeast system.[1,2]

Recently, however, our study of autophagy of invading bacteria revealed that the mammalian autophagy system is partially different from the yeast system.[3] In this review, we summarize the current understanding of how the mammalian ATG proteins behave during autophagosome formations, especially focusing on the LC3 proteins, well known markers of autophagosomes in mammalian cells.

## AUTOPHAGY IN THE LIVER

A PHYSIOLOGICAL FUNCTION of autophagy under starvation conditions in liver tissue has been reported. Mice with deficient autophagy specifically in the liver die more quickly than wild type (WT) mice in starvation conditions due to low levels of gluconeogenesis,[4] which result from lack of energy that is usually supplied by autophagy. This indicates that autophagy plays a critical function in hepatic cells in energy

*Correspondence: Professor Tamotsu Yoshimori, Laboratory of Intracellular Membrane Dynamics, Graduate School of Frontier Biosciences, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Email: tamyoshi@fbs.osaka-u.ac.jp*
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homeostasis under starvation conditions. On the other hand, a function of autophagy under steady state conditions has also been reported. Liver-specific autophagy-deficient mice show dramatic enlargement of the liver, and present with adenoma.[4] The liver tissue of these mice shows an accumulation of inclusion bodies similar to the aggregates frequently identified in human hepatocellular carcinomas (HCC). These results suggest that basal autophagy plays an indispensable role in cellular homeostasis by clearing inclusion bodies and protein aggregates. Similar results have been reported using mice with systemic or chimeric deficiency of autophagy.[5] Interestingly, tumor formation in these mice has been observed only in the liver tissue, suggesting that autophagy has a critical function in suppressing spontaneous tumorigenesis, especially in the liver.

Another aspect of the involvement of autophagy in liver disease is hepatitis C virus (HCV) infection. There are more than 180 million patients in the world with chronic HCV infection, and about 75% of HCC is caused by HCV infection.[6] Recently, several independent groups reported the function of autophagy in HCV life cycle and pathogenesis. Common observations from these reports indicate that autophagosome formation is significantly upregulated in HCV-infected cells.[7–11] Interestingly, this upregulation is dependent on the cellular unfolded protein response (UPR), which is usually induced by endoplasmic reticulum (ER) stress. HCV infection efficiently activates the UPR, and induced autophagy is attenuated by depleting the factors involved in the UPR.[11] Furthermore, several groups reported that induction of autophagy suppressed the innate immune response and resulted in the upregulation of viral replication.[12–14] Though the detailed mechanism is still unclear, autophagy may be a therapeutic target for controlling HCV infection.

## PROTEIN FACTORS INVOLVED IN AUTOPHAGOSOME FORMATION

GENES INVOLVED IN autophagosome formation have been identified by two different analyses in yeast. Treatment of yeast with vacuolar proteinase inhibitors leads to the massive accumulation of "autophagic bodies," which are derived from cytoplasmic contents sequestered by autophagy.[15] In cells lacking autophagy, however, autophagic bodies are never detected in the vacuole.[15] In the early 1990s, Yoshinori Ohsumi's group performed an extensive genetic screen against a yeast single-gene-deletion library to search for mutant cells that had empty vacuoles even in the presence of proteinase inhibitors.[16] At almost the same time, Daniel Klionsky's group performed a yeast genetic analysis focusing on the Cvt (cytoplasm-to-vacuole targeting) pathway, which mediates the biosynthetic transport of the vacuolar protein aminopeptidase 1 (Ape1) from the cytoplasm to the vacuole.[17] Interestingly, by screening for deficiencies in the Cvt pathway, they also isolated genes involved in autophagy.[18] From these screens, 18 genes were identified as core ATG genes; some of these are conserved in mammalian cells. The ATG gene products are currently thought to be involved in membrane dynamics during autophagosome formation. A series of biochemical analyses subsequently classified the 18 *ATG* proteins into six functional subgroups: (i) the ULK1 kinase and its regulatory complex; (ii) the ATG14L complex containing PI3K (the phosphoinositide 3-kinase complex); (iii) the WIPI-ATG2, PI3P (Phosphatidylinositol 3-phosphate) binding complex; (iv) the ATG9 subfamily; (v) the ATG12 UBL (ubiquitin-like) conjugation system; and (vi) the LC3 UBL and PE (phosphatidylethanolamine) conjugation system. These six functional complexes are thought to be involved in membrane extension, curvature formation, fusion, and fission during autophagosome formation. Detailed information regarding the function of each sub-complex is provided in several other nice reviews; therefore, we will only summarize briefly below.

The ULK1/2 kinase complex is an approximately 3 MDa complex, which includes the ULK1/2 kinase, ATG13, FIP200, and ATG101.[19–22] In both yeast and mammalian cells, starvation induced autophagy is under the control of the Tor (target of rapamycin) kinase.[19] In nutrient-rich conditions, the ULK complex is phosphorylated by mTOR complex 1 (mTORC1); however, in starvation conditions, nutrient sensor signals downregulate mTORC1 activity and the ULK1 complex is dephosphorylated. This dephosphorylation activates ULK1 and triggers activation of the autophagic pathway.

The ATG14L complex consists of four subunits, VPS34, VPS15, Beclin1, and ATG14L.[23,24] VPS34 is the PI3-kinase subunit, and generates phosphatidylinositol 3-phosphate at local membrane sites. PI3P plays an important role in the assembly of downstream effector molecules such as the WIPI family and DFCP1.[25–28] PI3-kinase activity is essential for autophagosome formation, as small molecule inhibitors like 3-methyladenine and wortmannin block the autophagic pathway.[29] The ATG14L subunit functions to recruit downstream ATG proteins to the ER membrane;[30] however, the molecular

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homeostasis under starvation conditions. On the other hand, a function of autophagy under steady state conditions has also been reported. Liver-specific autophagy-deficient mice show dramatic enlargement of the liver, and present with adenoma.[4] The liver tissue of these mice shows an accumulation of inclusion bodies similar to the aggregates frequently identified in human hepatocellular carcinomas (HCC). These results suggest that basal autophagy plays an indispensable role in cellular homeostasis by clearing inclusion bodies and protein aggregates. Similar results have been reported using mice with systemic or chimeric deficiency of autophagy.[5] Interestingly, tumor formation in these mice has been observed only in the liver tissue, suggesting that autophagy has a critical function in suppressing spontaneous tumorigenesis, especially in the liver.

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mechanism by which ATG14L itself is recruited to the membrane remains unknown.

WIPI–ATG2 complexes are downstream effectors of PI3P, and are recruited to PI3P-enriched sites by direct binding to PI3P.[26] These complexes consists of two sub-families, the WIPI family and the ATG2 family. To date, four WIPI sub-family proteins (WIPI-1 to –4)[26,31] and two ATG2 sub-family proteins (ATG2A and ATG2B) have been identified in humans, and all WIPI proteins can bind to both ATG2A and ATG2B (Morita *et al.*, unpubl. data, 2011), resulting in eight different combinations. WIPI proteins have WD40 repeat domains that can bind to both PI3P and ubiquitin. The ATG2 proteins weigh 180 kDa, but no obvious protein motifs have been identified, and their function is unclear.

ATG9 is a six-transmembrane protein. In yeast, ATG9 mainly localizes at small cytoplasmic dot-like structures that are recruited to the autophagosome initiation site (termed the PAS, pre-autophagosomal membrane/phagofore assembly site; see below) under starvation conditions.[32] Cytoplasmic ATG9 may exist on small membrane vesicles, and the fusion of ATG9-positive vesicles under starvation conditions may be related to the extension of the isolation membrane.[33] In mammals, two ATG9 family proteins (ATG9A and ATG9B) have been identified and mainly localize at the Golgi apparatus; the meaning of the Golgi localization is unknown.[33,34] The localization of yeast ATG9 to the PAS is dependent on the ATG1 complex, and ATG9 directly interacts with ATG17, one of the subunits of the yeast ATG1 complex. However, ATG9 localization is not affected by deficiencies of the ATG12 or ATG8 conjugation systems (see below).[35] Therefore, it is thought that ATG9 functions just downstream of the ATG1 complex, but upstream of the ATG12 and ATG8 conjugation systems (Fig. 1).

Among the ATG proteins, two different families of ubiquitin-like (UBL) proteins are involved in autophagosome formation: ATG12 and LC3 family proteins.[36,37] Almost half of all ATG proteins are involved in the conjugation systems for these UBLs. ATG12 is conjugated to the amide moiety of lysine 130 of ATG5 via an isopeptide bond. This reaction is catalyzed by two enzymes, ATG7 and ATG10, which correspond to the E1 and E2 enzymes of the ubiquitin conjugation system. ATG12–ATG5 forms a stable complex with ATG16L1 or

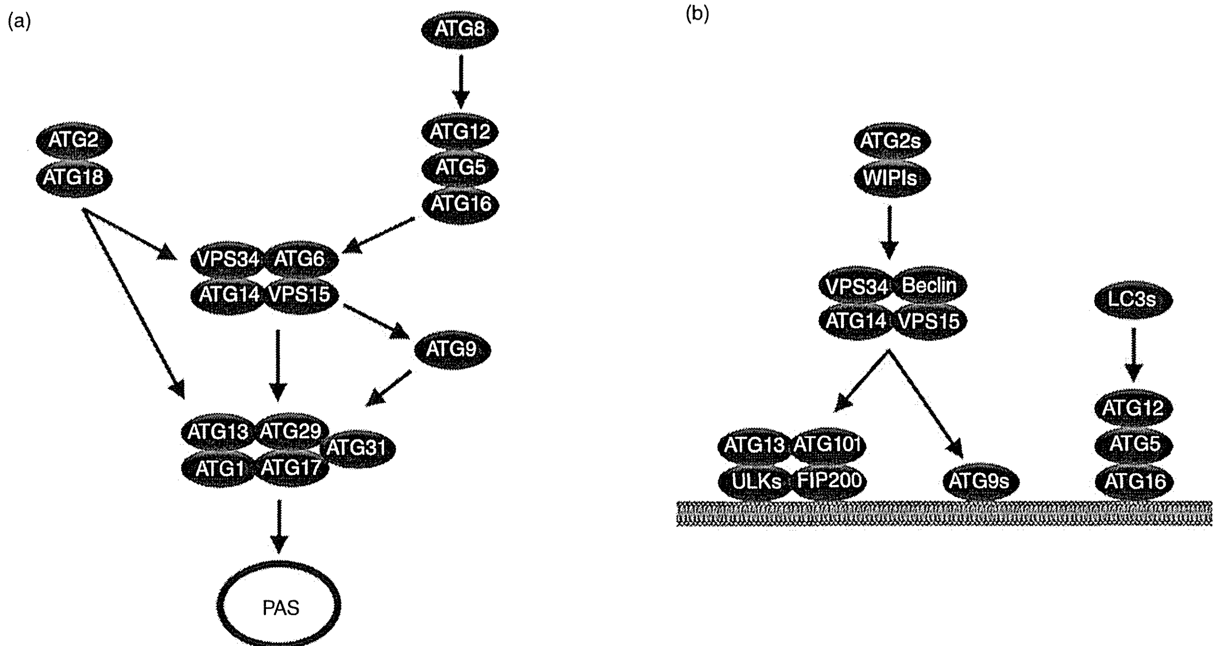


**Figure 1** Hierarchical relationships among the autophagy (ATG) proteins during recruitment. When upstream ATG protein function is deficient, the localization of downstream ATG proteins is lost. These schemas indicate the localization of ATG proteins to the pre-autophagosomal membrane/phagofore assembly site (PAS) during starvation-induced autophagy in yeast (a), or to the membrane in autophagy of invading bacteria in mammalian cells (b).

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ATG16L2, and functions in ATG8–PE conjugation.[38] LC3s, the mammalian homologs of yeast ATG8, consist of multiple protein families (LC3A, LC3B, LC3C, GABARAP, GATE-16, ATG8L) in the human genome.[39] All of these proteins are processed at their C-terminal residues by specific cysteine proteinases, ATG4s, that are encoded by four different genes (ATG4A-D) in humans.[40] Subsequently, PE is conjugated to the exposed C-terminal glycine residue of LC3 by the catalytic enzymes ATG7 and ATG3, which correspond to the E1 and E2 enzymes of the ubiquitin conjugation system, and LC3-PE is anchored to the membrane.[37,39] The ATG12–ATG5–ATG16L complex is thought to recruit PE conjugated LC3 to the site of autophagosome formation.[41] Lipidated LC3s are the final products of the ATG reaction cascade, and LC3 puncta formation is widely used as a marker for autophagosome formation.[29]

## SEQUENTIAL MEMBRANE RECRUITMENT OF ATG COMPLEXES

THE ORDER OF recruitment of the ATG complexes to the site of autophagosome formation has been analyzed in the yeast system.[35] In yeast, all of the ATG proteins localize at a peri-vacuolar dot structure termed the PAS, which is thought to be the point of origin of the autophagosome. The detailed characteristics of the PAS have not yet been elucidated.[42] Importantly, the PAS localization of each ATG protein is dependent on another ATG protein. These observations allow us to determine hierarchical relationships among the ATG proteins during autophagosome formation (summarized in Figure 1 left panel). [2,35] For example, in cells lacking the ATG1 complex, almost all of the other ATG proteins lose PAS localization, suggesting that the ATG1 complex plays an important role in the initiation of assembly of the ATG proteins at the PAS. On the other hand, lack of ATG8 does not affect the PAS localization of any other ATG protein, indicating that ATG8 may function at the downstream end of this pathway. Similar types of experiments in mammalian cells have shown that ATG proteins form dot structures close to the ER membrane under starvation conditions. Though there is no evidence that these structures correspond to the yeast PAS, the hierarchical relationships found in yeast seem to be conserved in mammalian cells.

However, several differences in the relationships of the ATG complexes have been identified during the process of selective autophagy in mammalian cells. Recently, our group performed a systematic analysis of ATG complex recruitment to the membrane during autophagy of bacteria.[3] *Salmonella enterica* serovar typhimurium is a Gram-negative bacterium that can invade epithelial cells in a single membrane organelle called a Salmonella-containing vacuole (SCV).[43,44] Invading bacteria can be surrounded by autophagosomes in a selective process and killed by lysosomal hydrolases; thus, autophagy functions to protect host cells from pathogenic bacteria as an intracellular innate immune system.[45] During this process, almost all of the ATG proteins are recruited to the membrane around the invading bacterium. We examined the localization of all ATG complexes to the bacterial membrane in a series of MEF (mouse embryonic fibroblasts) cells derived from several different ATG knockout mice, and showed that the hierarchical relationships of the ATG complexes during membrane recruitment are different from those during starvation-induced autophagy in yeast cells (summarized in Figure 1 right panel).[3]

## THE LC3-POSITIVE COMPARTMENT DOES NOT ALWAYS REPRESENT THE ISOLATION MEMBRANE/AUTOPHAGOSOME

ONE OF THE important observations from our study is that three ATG complexes (the ATG1, ATG9, and ATG16L complexes) are recruited to the membrane independently.[3] Interestingly, LC3 proteins are recruited to the invading bacteria even in the absence of ATG1, ATG9L1, or ATG14L, indicating that LC3s can be recruited to the membrane without autophagosome formation. These results are quite different from the model of PAS recruitment in yeast. We do not understand why ATG complexes show such different behavior in each system; however, it may be explained by the differences between the selective and non-selective pathways.

Though the canonical relationships among ATG complexes cannot be adapted to autophagy of bacteria, a similar phenomenon has been reported in the yeast system. A systematic analysis of ATG8–PE conjugation (ATG8 was named Aut7p in this paper) reactions in ATG-deficient cells showed that ATG8–PE conjugation is blocked only in ATG3-, ATG4-, ATG5-, ATG7-, ATG10-, ATG12-, and ATG16-deficient cells, but not in ATG1-, ATG2-, ATG6-, ATG9-, ATG13-, ATG14-, and ATG17-deficient cells.[35] The latter mutants lack functional autophagy, but show the same ATG8–PE levels as WT cells, indicating that ATG8–PE formation does not always correlate with autophagy function.

Thus, although LC3–PE formation can be a good marker of autophagosome formation, LC3–PE

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conjugation can be induced independent of autophagosome formation in some contexts, and the LC3 positive compartment does not always represent isolation membranes.

## LC3 IS NOT REQUIRED FOR FORMATION OF DOUBLE-MEMBRANE STRUCTURES OR TARGET RECOGNITION DURING AUTOPHAGY OF BACTERIA

IN ADDITION TO the results discussed above, we observed that cytoplasmic invading bacteria were positive for ATG5 and were surrounded by double-membrane structures even in ATG3 knockout cells, which are deficient for the LC3–PE conjugation reaction.[3] These results indicate that neither LC3–PE conjugation nor LC3–PE recruitment to the membrane are required for the formation and elongation of the isolation membrane during autophagy of bacteria.

Additional study is needed to determine whether this phenotype occurs commonly in non-selective autophagy; however, similar results have been reported from the study of starvation-induced autophagy in mammalian cells. In ATG3 knockout cells or catalytic domain-inactive ATG4B mutant-expressing cells, double-membrane structures were induced efficiently under starvation conditions despite a complete block of LC3–PE conjugation.[46,47] These results suggest that LC3–PE conjugation may not be required for the formation of the isolation membrane in both non-selective and selective autophagy. Interestingly, the majority of isolation membranes accumulated in ATG3-deficient or ATG4B mutant-expressing cells appear to be unclosed and incomplete autophagosomes,[46,47] suggesting that LC3 functions in the final step of autophagosome formation, by closing the double-membrane structure to separate the internal vesicle from the outer membrane.

Recently, an adaptor protein model has been proposed for target recognition during selective autophagy. This model is based on the idea that adapter proteins, such as p62, NBR1, NDP52, and OPTN, recognize both ubiquitinated targets and LC3 proteins, and function to bridge the targets and LC3 proteins on the isolation membrane.[48–52] This model indicates that LC3 proteins have essential roles in target recognition during selective autophagy. However, the fact that LC3 is not necessary for the formation of isolation membranes during sequestration of target proteins indicates that the adaptor protein model is not an essential pathway in selective autophagy.

How does the isolation membrane first recognize the target protein in selective autophagy? We cannot fully explain this yet, but upstream ATG complexes, such as ATG1, ATG9, and ATG14L complexes, may be involved prior to LC3 recruitment, because the deficiencies in these complexes are critical in the formation of isolation membranes and elimination of invading bacteria from the cell.[3]

Recently, we performed extensive yeast two-hybrid and IP-mass spec analyses to generate a direct interaction network for the mammalian ATG complexes and identified several new protein–protein interactions that may link the entire protein network together. This effort has led to a new model for understanding the entire molecular machinery of the mammalian autophagic pathway.

## CONCLUSION

THERE ARE MANY remaining questions regarding the molecular mechanisms of mammalian selective autophagy, including (i) how the autophagy recognizes unwanted substrates that have accumulated in the cytoplasm? (ii) How does each ATG complex physically interact with the other complexes, and function in the membrane dynamics of autophagosome formation? (iii) What is the function of LC3? The molecular mechanisms of mammalian autophagy are currently thought to be similar to the yeast system. However, our recent study of autophagy of invading bacteria has demonstrated that the hierarchical relationships in membrane recruitment of ATG complexes during mammalian selective autophagy is not identical to that of recruitment to the yeast PAS. Further analysis is needed to more fully understand the pathway of mammalian selective autophagy.

LC3-PE formation has been widely used as a marker of autophagosome formation, and has contributed to our understanding of the physiological roles of autophagy in various biological processes. However, our recent findings show that LC3 puncta formation is induced independently of autophagosome formation in some contexts. This alerts us that monitoring LC3 behavior is not enough to judge the induction of autophagy.

## REFERENCES

1 Mizushima N, Yoshimori T, Ohsumi Y. The role of ATG proteins in autophagosome formation. *Annu Rev Cell Dev Biol* 2010; 27: 107–32.

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2 Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y. Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nat Rev Mol Cell Biol* 2009; **10**: 458–67.
3 Kageyama S, Omori H, Saitoh T *et al.* The LC3 recruitment mechanism is separate from Atg9L1-dependent membrane formation in the autophagic response against Salmonella. *Mol Biol Cell* 2011; **22**: 2290–300.
4 Komatsu M, Waguri S, Ueno T *et al.* Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol* 2005; **169**: 425–34.
5 Takamura A, Komatsu M, Hara T *et al.* Autophagy-deficient mice develop multiple liver tumors. *Genes Dev* 2011; **25**: 795–800.
6 Moradpour D, Penin F, Rice CM. Replication of hepatitis C virus. *Nat Rev Microbiol* 2007; **5**: 453–63.
7 Ait-Goughoulte M, Kanda T, Meyer K, Ryerse JS, Ray RB, Ray R. Hepatitis C virus genotype 1a growth and induction of autophagy. *J Virol* 2008; **82**: 2241–9.
8 Tanida I, Fukasawa M, Ueno T, Kominami E, Wakita T, Hanada K. Knockdown of autophagy-related gene decreases the production of infectious hepatitis C virus particles. *Autophagy* 2009; **5**: 937–45.
9 Dreux M, Gastaminza P, Wieland SF, Chisari FV. The autophagy machinery is required to initiate hepatitis C virus replication. *Proc Natl Acad Sci U S A* 2009; **106** (33): 14046–51.
10 Taguwa S, Kambara H, Fujita N *et al.* Dysfunction of autophagy participates in vacuole formation and cell death in cells replicating hepatitis C virus. *J Virol* 2011; **85** (24): 13185–94.
11 Sir D, Chen W-L, Choi J, Wakita T, Yen TSB, Ou J-HJ. Induction of incomplete autophagic response by hepatitis C virus via the unfolded protein response. *Hepatology* 2008; **48**: 1054–61.
12 Estrabaud E, De Muynck S, Asselah T. Activation of unfolded protein response and autophagy during HCV infection modulates innate immune response. *J Hepatol* 2011; **55**: 1150–3.
13 Ke P-Y, Chen SS-L. Activation of the unfolded protein response and autophagy after hepatitis C virus infection suppresses innate antiviral immunity in vitro. *J Clin Invest* 2011; **121**: 37–56.
14 Shrivastava S, Raychoudhuri A, Steele R, Ray R, Ray RB. Knockdown of autophagy enhances the innate immune response in hepatitis C virus-infected hepatocytes. *Hepatology* 2011; **53**: 406–14.
15 Baba M, Takeshige K, Baba N, Ohsumi Y. Ultrastructural analysis of the autophagic process in yeast: detection of autophagosomes and their characterization. *J Cell Biol* 1994; **124**: 903–13.
16 Tsukada M, Ohsumi Y. Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. *FEBS Lett* 1993; **333** (1-2): 169–74.
17 Baba M, Osumi M, Scott SV, Klionsky DJ, Ohsumi Y. Two distinct pathways for targeting proteins from the cytoplasm to the vacuole/lysosome. *J Cell Biol* 1997; **139**: 1687–95.
18 Harding TM, Hefner-Gravink A, Thumm M, Klionsky DJ. Genetic and phenotypic overlap between autophagy and the cytoplasm to vacuole protein targeting pathway. *J Biol Chem* 1996; **271** (30): 17621–4.
19 Hosokawa N, Hara T, Kaizuka T *et al.* Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol Biol Cell* 2009; **20**: 1981–91.
20 Ganley IG, Lam DH, Wang J, Ding X, Chen S, Jiang X. ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. *J Biol Chem* 2009; **284** (18): 12297–305.
21 Hosokawa N, Sasaki T, Iemura S-I, Natsume T, Hara T, Mizushima N. Atg101, a novel mammalian autophagy protein interacting with Atg13. *Autophagy* 2009; **5**: 973–9.
22 Jung CH, Jun CB, Ro S-H *et al.* ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell* 2009; **20**: 1992–2003.
23 Matsunaga K, Saitoh T, Tabata K *et al.* Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nat Cell Biol* 2009; **11**: 385–96.
24 Itakura E, Kishi C, Inoue K, Mizushima N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. *Mol Biol Cell* 2008; **19**: 5360–72.
25 Taguchi-Atarashi N, Hamasaki M, Matsunaga K *et al.* Modulation of local PtdIns3P levels by the PI phosphatase MTMR3 regulates constitutive autophagy. *Traffic* 2010; **11**: 468–78.
26 Polson HEJ, de Lartigue J, Rigden DJ *et al.* Mammalian Atg18 (WIPI2) localizes to omegasome-anchored phagophores and positively regulates LC3 lipidation. *Autophagy* 2010; **6**: 506–522.
27 Axe EL, Walker SA, Manifava M *et al.* Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J Cell Biol* 2008; **182**: 685–701.
28 Proikas-Cezanne T, Ruckerbauer S, Stierhof Y-D, Berg C, Nordheim A. Human WIPI-1 puncta-formation: a novel assay to assess mammalian autophagy. *FEBS Lett* 2007; **581**: 3396–404.
29 Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. *Cell* 2010; **140**: 313–26.
30 Matsunaga K, Morita E, Saitoh T *et al.* Autophagy requires endoplasmic reticulum targeting of the PI3-kinase complex via Atg14L. *J Cell Biol* 2010; **190**: 511–21.
31 Jeffries TR, Dove SK, Michell RH, Parker PJ. PtdIns-specific MPR pathway association of a novel WD40 repeat protein, WIPI49. *Mol Biol Cell* 2004; **15**: 2652–63.
32 Noda T, Kim J, Huang WP *et al.* Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation


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in the Cvt and autophagy pathways. *J Cell Biol* 2000; **148**: 465–80.

33 Young ARJ, Chan EYW, Hu XW *et al.* Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. *J Cell Sci* 2006; **119** (Pt 18): 3888–900.

34 Yamada T, Carson AR, Caniggia I *et al.* Endothelial nitric-oxide synthase antisense (NOS3AS) gene encodes an autophagy-related protein (APG9-like2) highly expressed in trophoblast. *J Biol Chem* 2005; **280**: 18283–90.

35 Suzuki K, Kirisako T, Kamada Y, Mizushima N, Noda T, Ohsumi Y. The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *EMBO J* 2001; **20** (21): 5971–81.

36 Mizushima N, Noda T, Yoshimori T *et al.* A protein conjugation system essential for autophagy. *Nature* 1998; **395** (6700): 395–8.

37 Ichimura Y, Kirisako T, Takao T *et al.* A ubiquitin-like system mediates protein lipidation. *Nature* 2000; **408** (6811): 488–92.

38 Hanada T, Noda NN, Satomi Y *et al.* The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. *J Biol Chem* 2007; **282** (52): 37298–302.

39 Kabeya Y, Mizushima N, Ueno T *et al.* LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 2000; **19**: 5720–8.

40 Kirisako T, Ichimura Y, Okada H *et al.* The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *J Cell Biol* 2000; **151**: 263–76.

41 Fujita N, Itoh T, Omori H, Fukuda M, Noda T, Yoshimori T. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Mol Biol Cell* 2008; **19**: 2092–100.

42 Suzuki K, Ohsumi Y. Current knowledge of the pre-autophagosomal structure (PAS). *FEBS Lett* 2010; **584**: 1280–6.

43 Finlay BB, Brumell JH. Salmonella interactions with host cells: *in vitro* to *in vivo*. *Philos Trans R Soc Lond B Biol Sci* 2000; **355** (1397): 623–31.

44 Haraga A, Ohlson MB, Miller SI. Salmonellae interplay with host cells. *Nat Rev Microbiol* 2008; **6**: 53–66.

45 Birmingham CL, Smith AC, Bakowski MA, Yoshimori T, Brumell JH. Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. *J Biol Chem* 2006; **281** (16): 11374–83.

46 Fujita N, Hayashi-Nishino M, Fukumoto H *et al.* An Atg4B mutant hampers the lipidation of LC3 paralogues and causes defects in autophagosome closure. *Mol Biol Cell* 2008; **19** (11): 4651–9.

47 Sou Y-S, Waguri S, Iwata J-I *et al.* The Atg8 conjugation system is indispensable for proper development of autophagic isolation membranes in mice. *Mol Biol Cell* 2008; **19** (11): 4762–75.

48 Bjørkøy G, Lamark T, Brech A *et al.* p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol* 2005; **171**: 603–14.

49 Komatsu M, Waguri S, Koike M *et al.* Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. *Cell* 2007; **131**: 1149–63.

50 Kirkin V, Lamark T, Sou Y-S *et al.* A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. *Mol Cell* 2009; **33**: 505–16.

51 Thurston TLM, Ryzhakov G, Bloor S, von Muhlinen N, Randow F. The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. *Nat Immunol* 2009; **10**: 1215–21.

52 Wild P, Farhan H, McEwan DG *et al.* Phosphorylation of the autophagy receptor optineurin restricts Salmonella growth. *Science* 2011; **333** (6039): 228–33.

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MINIREVIEW

# Differential requirements of mammalian ESCRTs in multivesicular body formation, virus budding and cell division

Eiji Morita

Laboratory of Viral Infection, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Japan

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**Correspondence**
E. Morita, Laboratory of Viral Infection, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita 565-0871, Osaka, Japan
Fax: +81 6 6879 4252
Tel: +81 6 6879 8335
E-mail: moritae@biken.osaka-u.ac.jp

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The endosomal sorting complexes required for transport (ESCRTs) mediate membrane fission from the cytoplasmic face of the bud neck. ESCRTs were originally identified as factors involved in multivesicular body vesicle biogenesis in yeast but have since been shown to function in other membrane fission events in mammalian cells, including enveloped virus budding and the abscission step of cytokinesis. Several recent studies have revealed that not all ESCRT factors are required for each of these biological processes, and this review summarizes our current understanding of the different requirements for ESCRT factors in these three different ESCRT-mediated mammalian membrane fission processes.

## Introduction

Integral membrane proteins, such as receptors and ion channels, are ultimately degraded within lysosomes. During this process, cargos destined for degradation are removed from limiting endosomal membranes by sorting into intralumenal vesicles (ILVs) that bud into the interior of multivesicular bodies (MVBs)/late endosomes. Screening from a yeast single deletion mutant library identified a set of approximately 20 proteins that drive formation of ILVs at the MVB [1,2]. These proteins form multiprotein complexes named endosomal sorting complexes required for transport (ESCRTs) [3]. These factors are well conserved in higher eukaryotes and are encoded by multiple genes; the number of factors thus far identified has expanded to more than 30 in humans [1,2]. The role of the ESCRT machinery in higher eukaryotic cells extends to other membrane fission events, including enveloped viral budding and abscission of cytokinesis. All of these events require topologically similar membrane transformations that require membrane constriction and fission from the cytosolic face of the bud neck [1,2,4].

ESCRTs can be classified into six mono- or hetero-oligomeric complexes (ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III, ALIX and VPS4). These complexes are

**Abbreviations**
AAA-ATPase, ATPase associated with a variety of cellular activities; CHMP, charged MVB protein; EGFR, endothelial growth factor receptor; ESCRT, endosomal sorting complex required for transport; ILV, intralumenal vesicle; MIT, microtubule interacting and transport; MVB, multivesicular body; PRR, proline-rich region; RNAi, RNA interference.

recruited sequentially to membranes and function in protein sorting, membrane remodeling and fission. The role and action of the ESCRTs in membrane dynamics has been proposed from both *in vivo* and *in vitro* study of yeast systems [5–7]. At the first step, early-acting ESCRTs such as ESCRT-I and ESCRT-II interact with upstream recruiting factors, such as ESCRT-0, and these factors have an important role in the concentration of cargo proteins and assist in the deformation of membranes. These early-acting factors function to recruit late-acting ESCRTs, subunits of the ESCRT-III complex, which assemble like filaments within the necks of membrane tubules and mediate membrane fission. ESCRT-III assemblies, in turn, recruit VPS4 ATPases, which use the energy of ATP hydrolysis to disassemble the ESCRT complexes.

ESCRTs have been considered to employ similar mechanisms for their roles in each of the three biological processes mentioned above: MVB vesicle formation, virus budding, and cytokinesis. However, several recent studies have suggested that only subsets of ESCRTs are required to complete membrane fission in some specific pathway. Therefore, this review will describe the recent findings on the differential requirements of mammalian ESCRTs in the three biological processes of MVB vesicle formation, virus budding, and cytokinesis.

## ESCRT recruitment

The ESCRT pathway must function at different cellular membranes, which implies that there must be membrane-specific adaptor complexes that recruit the pathway to the proper membrane at the proper time. The HIV-1 structural protein Gag contains two distinct late domains that are essential for the detachment of budding particles from the cell surface. These late domains recruit cellular ESCRTs that complete the budding process. The P(T/S)AP late domain recruits ESCRT-I via its interaction with the TSG101 subunit, and the YP(X)nL late domain recruits ALIX [8,9]. Both ESCRT-I and ALIX (called early-acting ESCRTs) in turn recruit downstream ESCRT-III (a late-acting ESCRT) that facilitates membrane fission and virus release. These late domains are identified in several different viral structural proteins, and the ESCRT pathway is thought to be a common machinery for the budding process in several different enveloped RNA viruses [4].

The P(T/S)AP late domain was also identified in the cellular protein HRS, which forms a stable complex with STAM1 or STAM2. The PSAP motif in HRS has been shown to recruit ESCRT-I through its direct interaction with the TSG101 subunit, and this interaction is critical for cargo protein degradation at the lysosome [10–13]. Thus, HRS has been considered to be a cellular counterpart of Gag and to function as an ESCRT recruiter for the MVB pathway. The P(T/S)AP late domain has also been identified in two other endosomal proteins, GGA3 and TOM1L1 [14,15]. The primary structures of GGA3 and TOM1L1 are similar to that of HRS: both contain VHS (Vps27, His, STAM) and GAT (GGA and TOM) domains, directly interact with TSG101 via its P(T/S)AP motifs and function as ESCRT-I recruiters like HRS. So far, there is no strong evidence of functional differences among these endosomal proteins, but it is very likely that the proteins recognize different types of cargo proteins to deliver to the lysosome by using the same ESCRT machinery.

In the case of cytokinesis, CEP55 functions as an ESCRT recruiter. CEP55 was originally identified as a protein localized at a centrosome during interface and at a Fleming body (center of the midbody) during cytokinesis, and CEP55 is considered to have a role in the coordination of mitosis and cytokinesis [16]. In screens for binding partners of early-acting ESCRTs, CEP55 was identified as a direct binding partner for both TSG101 (ESCRT-I) and ALIX [17]. The CEP55 binding region of both TSG101 and ALIX is located in the proline-rich region (PRR), and CEP55 binds both proteins in a similar fashion [18]. So far, no CEP55 paralogs have been identified, but it would not be surprising if an alternative factor was found to function in cytokinesis because the effect of RNA interference (RNAi) depletion is seen only in limited types of cultured cells.

Thus, three different sets of ESCRT recruiting factors function in each of the three ESCRT-mediated biological processes, either by recognizing target proteins or by recruiting soluble downstream factors to the site of the budding neck (Fig. 1).

## ESCRT-I

Soluble ESCRT-I complexes contain single copies of each of the four different subunit types (TSG101, VPS28, VPS37 and MVB12) [19,20]. In humans, multiple VPS37 (A–D) and MVB12 (A and B) paralogs can combine to produce up to eight different ESCRT-I variants [19]. RNAi depletion of TSG101, a unique ESCRT-I subunit, inhibits all ESCRT-mediated biological processes, indicating its essential roles in all pathways [8,12,17,21]. However, several studies have suggested that the eight different ESCRT-I variants are functionally diverse. Simultaneous RNAi depletion

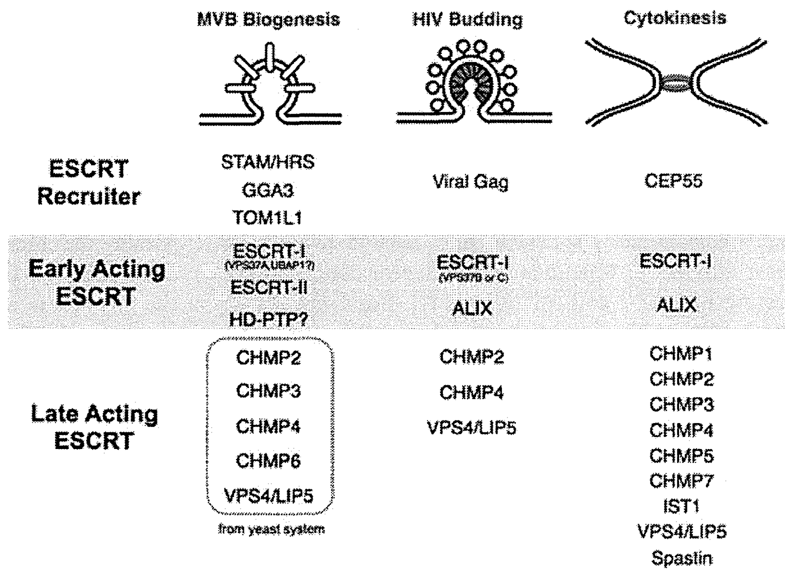


**Fig. 1.** Factor requirements for ESCRT-mediated biological processes. The list indicates proteins that are known to be involved in MVB biogenesis, virus budding and cytokinesis. The list of late-acting ESCRTs for MVB biogenesis was derived from analyses in yeast, because of the lack of systematic analyses of the effects of their RNAi depletion in mammalian cells.

of VPS37B and VPS37C dramatically reduces virus budding, although individual RNAi depletion of VPS37B or VPS37C has no effects on such budding [22]. These results suggest that virus budding requires at least VPS37B- or VPS37C-containing complexes, and these complexes can function redundantly. On the other hand, single depletion of VPS37A is enough to inhibit ligand-dependent endothelial growth factor receptor (EGFR) degradation [23], suggesting that only VPS37A-containing complexes have an essential role in MVB vesicle formation. Similar results have been reported for MVB12 subunits. Co-depletion, but not single depletion, of MVB12A and MBV12B inhibits infectivity of released HIV [19], while single depletion of MVB12A is enough to inhibit ligand-dependent EGFR degradation [24], suggesting that both MVB12A and MVB12B function in virus release but MVB12A plays the more critical role in MVB vesicle formation. These results suggest that ESCRT-I variants that contain different subunits of VPS37 or MVB12 may function in specific ESCRT-mediated processes, although systematic RNAi depletion analysis for all ESCRT-I subunits will be needed to completely clarify this matter. Little is known about the functional differences of ESCRT-I variants in cytokinesis, because the effect of RNAi depletion is very modest and it is very difficult to judge whether each ESCRT-I variant is involved in cytokinesis (Fig. 1).

Recently, another subunit, named UBAP1, was identified as a novel ESCRT-I subunit [25,26]. This subunit contains the UBA domain, which is also found in TSG101, and an MVB12-related sequence element. Interestingly, UBAP1 was found only in the VPS37A-containing complex and was shown to function in MVB formation but not in cytokinesis [25].

We are still lacking much information on the biological/biochemical characteristics of the eight different ESCRT-I variants, i.e. the expression patterns (tissue distributions), specific binding partners, membrane binding affinities, effects on the stability of the ESCRT-I complexes etc. This information would help to reveal how the different ESCRT-I variants function in each specific ESCRT-mediated pathway.

## ALIX

ALIX is recruited to the site of virus budding through the direct interaction of its V domain with the YP(X)nL late domain of viral Gag [9] or to the site of cytokinesis through direct interaction of its PRR with CEP55 [17,21]. In both cases, the N-terminal Bro domain of ALIX binds to CHMP4 proteins, and ALIX serves as an adaptor that recruits CHMP4/ESCRT-III proteins to function at distinct biological membranes [17,27]. ALIX is also known to interact with TSG101 (ESCRT-I) directly or indirectly [28,29]. However, ESCRT-I and ALIX can function independently, at least in some contexts. ALIX is known to be activated through a series of conformational changes and dimerization, and functions to nucleate the assembly of two strands of CHMP4 that form filaments within the necks.

So far, there is no clear evidence that ALIX is involved in MVB vesicle formation. However, another Bro domain protein, HD-PTP, was reported to have a specific function in MVB vesicle formation but not in

cytokinesis (Fig. 1) [30]. These results suggest that the different Bro domain proteins may have specific functions in the different biological processes.

## ESCRT-II

The ESCRT-II complex contains two copies of EAP20 and single copies of EAP30 and EAP45 [31,32]. In yeast, ESCRT-II functions as an adaptor complex connecting ESCRT-I and ESCRT-III [1]. Yeast ESCRT-II is recruited to the site of the membrane through direct interaction with ESCRT-I [33]. The NZF domain of Vps36 (human EAP45) interacts with Vps28 of ESCRT-I [34]. Then, ESCRT-II recruits two strands of ESCRT-III through direct interaction of Vps25 (human EAP20) with Vps20 (human CHMP6) [35]. In human cells, however, ESCRT-II is recruited to the site of the membrane in a different way, because the NZF domain is missing in EAP45. Several studies have shown a direct interaction between human ESCRT-I and ESCRT-II [36,37], but this interaction has not yet been fully characterized. ESCRT-II seems to be involved in MVB vesicle formation in human cells, because RNAi depletion of EAP45 blocks ligand-dependent EGFR degradation [36,38]. However, RNAi depletion of EAP45 does not have any effect on HIV budding or cytokinesis [36], suggesting that ESCRT-II has a specific function in MVB vesicle formation in human cells (Fig. 1).

**Table 1.** ESCRT factors. The names of complexes and their components in the yeast *Saccharomyces cerevisiae* and in humans are shown.

| Complex | Yeast proteins | Mammalian proteins |
|---|---|---|
| ESCRT-0 | Vps27 | Hrs |
| | Hse1 | STAM1, 2 |
| ESCRT-I | Vps23 | TSG101 |
| | Vps28 | VPS28 |
| | Vps37 | VPS37A, B, C, D |
| | Mvb12 | MVB12A, B |
| ESCRT-II | Vps22 | EAP30 |
| | Vps25 | EAP20 |
| | Vps36 | EAP45 |
| ESCRT-III | Vps2 | CHMP2A, B |
| | Vps24 | CHMP3 |
| | Vps20 | CHMP6 |
| | Snf7 | CHMP4A, B, C |
| | Vps60 | CHMP5 |
| | Did2 | CHMP1A, B |
| | – | CHMP7 |
| | Ist1 | IST1 |
| Others | Vps4 | VPS4A, B |
| | Vta1 | LIP5 |
| | Bro1 | ALIX, HD-PTP |
| | Doa4 | UBPY, AMSH |

## ESCRT-III

ESCRT-III subunits play a critical role in membrane fission reactions by assembling into helical filaments within the membrane neck structure. These higher order ESCRT-III assemblies are thought to constrict the membrane neck, ultimately leading to fission [6,7]. In yeast, ESCRT-III consists of four core subunits, Vps20, Snf7, Vps24 and Vps2, and three regulatory subunits, Did2, Vps60 and Ist1. All seven proteins seem to work together in MVB biogenesis, although deficiencies in the regulatory subunits produce only weak phenotypes [35,39,40]. A mechanistic model of the action of ESCRT-III in membrane fission has been proposed based on investigation in a yeast system both *in vivo* and *in vitro* [5–7]. First, two Vps20 subunits, which are recruited by ESCRT-II, appear to nucleate the assembly of two spiraling Snf7 filaments within the necks. Next, these filaments are capped by Vps24 and Vps2. Vps24 has the essential function of bridging Snf7 and Vps2. Finally, Vps2 recruits Vps4, and then the ESCRT factors are disassembled by its ATPase activity.

In humans, ESCRT-III proteins are called CHMPs (charged MVB proteins). The number of CHMP/ESCRT-III proteins has expanded from seven in yeast to 12 in humans (Table 1), including three isoforms of CHMP4 (yeast Snf7) and two each of CHMP1 (yeast Did2) and CHMP2 (yeast Vps2), and an additional family member, CHMP7, not found in yeast [2]. Due to the multiplicity of CHMP proteins in human cells, the overexpression of wild-type or truncated forms of CHMPs has frequently been utilized to examine the function of ESCRT-III. We recently performed systematic RNAi depletion analyses to determine the requirements for each of the CHMP proteins for both HIV budding and cytokinesis [41,42]. In these screens, we found that depletion of any of the 12 human CHMP proteins, with the possible exception of CHMP6, inhibited cytokinesis, and in most cases also gave rise to earlier defects in mitosis [42,43]. These results suggest that nearly all CHMP proteins play significant functional roles in cytokinesis and other stages of cell division. On the other hand, virus release was profoundly inhibited only when either CHMP2 or CHMP4 family members were co-depleted, and virus budding proceeded efficiently in the absence of CHMP1, CHMP3, CHMP5–7 and IST1 proteins [41,43]. These results indicate that efficient HIV-1 budding requires only a subset of CHMP proteins,