range and species tropism of the wbJOY_06-like HEV and to search for new HEV strains of unrecognized genotypes in humans and other animals.

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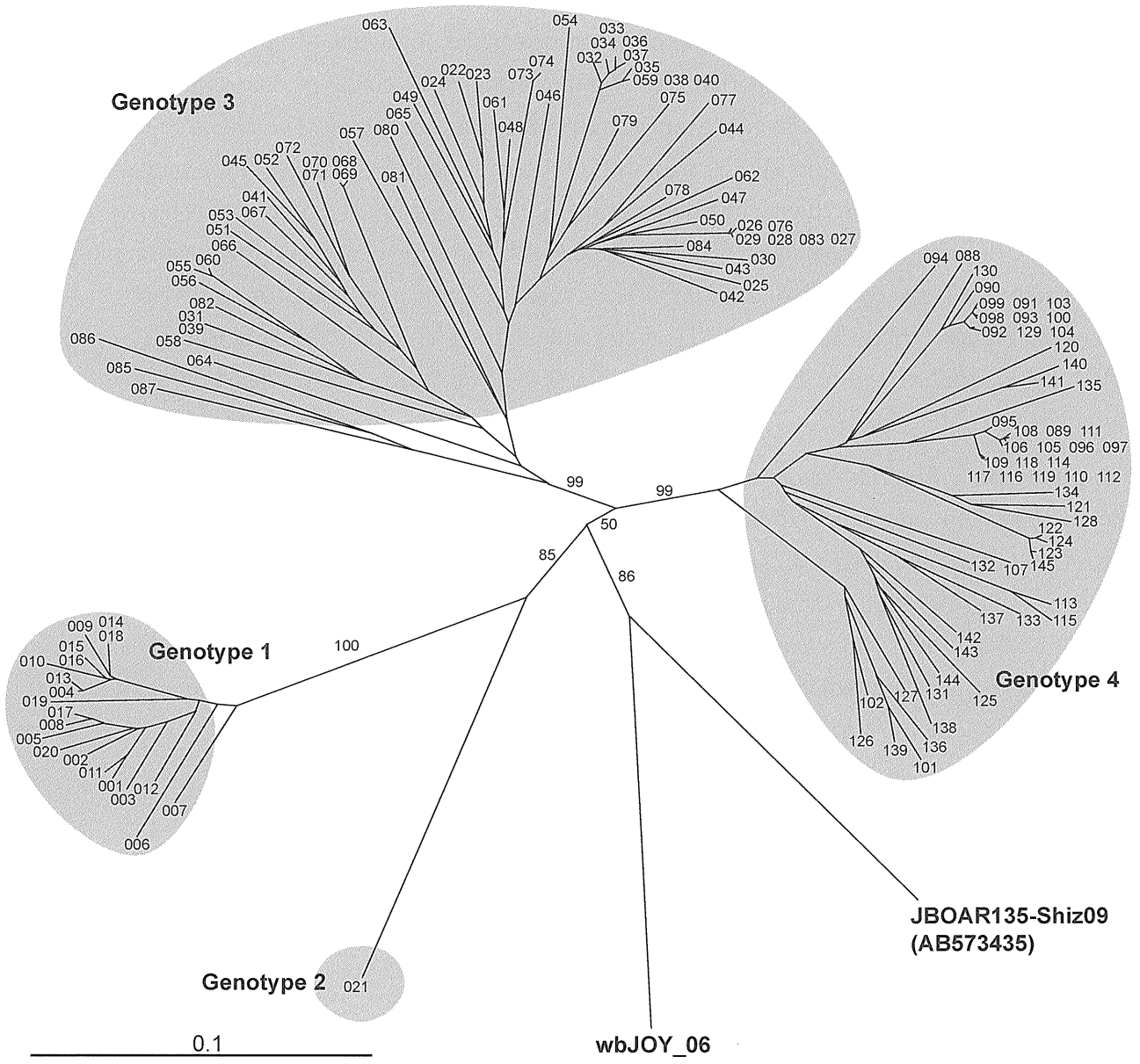
# Supplementary Fig. S1




# Supplementary Fig. S2

**Supplementary Fig. S1.** Complete genome scanning carried out by the Simplot software program for the wbJOY_06 versus 145 reported HEV isolates grouped by genotype, and avian HEV isolates ($n$=5) as an outgroup. (a) The results from a Simplot analysis are shown. The $y$-axis shows the percentage of identity within a sliding window 200 bp wide centred on the position plotted, with a step size between plots of 20 bp. (b) Bootscanning of the HEV sequence. The $y$-axis shows the percentage of permuted trees using a sliding window 200 bp wide centred on the position plotted, with a step size between plots of 20 bp. The open reading frame map is schematically shown at the top of the Figure. Abbreviations are: MT, methyltransferase; Y, Y domain; P, papain-like protease; HVR, hypervariable region; X, X domain; Hel, helicase; and RdRp, RNA-dependent RNA polymerase.

**Supplementary Fig. S2.** An unrooted phylogenetic tree constructed by the neighbour-joining method based on the partial 326 nt ORF1 sequence of 145 HEV isolates of genotypes 1-4 retrievable from the DDBJ/GenBank/EMBL database as of November 2010 (see Supplementary Table S2), the JBOAR135-Shiz09 isolate (AB573435) reported as 'genotype 5' (Takahashi *et al.*, 2010) and the wbJOY_06 isolate obtained in the present study. Both JBOAR135-Shiz09 and wbJOY_06 isolates are indicated in boldface type for visual clarity. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 resamplings.

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**Supplementary Table S1.** Comparison of the wbJOY_06 isolate of unclassifiable genotype with 152 reported mammalian and avian HEV isolates whose entire or near-entire sequence is known

| HEV isolate | No. of isolates compared | Divergence (%) [range (mean±SD)] |
|---|---|---|
| Mammalian | 147 | 53.8–77.4 (74.9±2.8) |
| Genotype 1 | 20 | 73.2–74.3 (74.0±0.3) |
| Genotype 2 | 1 | 72.9 |
| Genotype 3 | 66 | 72.3–74.8 (74.2±0.4) |
| Humans | 32 | 73.8–74.6 (74.2±0.2) |
| Swine | 22 | 74.0–74.8 (74.3±0.3) |
| Wild boars | 7 | 73.8–74.6 (74.1±0.3) |
| Deer | 1 | 74.3 |
| Mongoose | 1 | 74.4 |
| Rabbits | 3 | 72.3–72.7 (72.5±0.2) |
| Genotype 4 | 58 | 76.3–77.4 (76.8±0.2) |
| Humans | 41 | 76.4–77.4 (76.9±0.2) |
| Swine | 16 | 76.3–77.2 (76.8±0.3) |
| Wild boar | 1 | 76.8 |
| Rat | 2 | 53.8–54.0 (53.9±0.1) |
| Avian | 5 | 46.9–47.5 (47.2±0.2) |

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**Supplementary Table S2.** HEV isolates used for comparative and phylogenetic analyses in the present study

| Isolate no. | Genotype | Host | GenBank accession no. |
|---|---|---|---|
| 001 | 1 | Human | AF051830 |
| 002 | 1 | Human | AF076239 |
| 003 | 1 | Human | AF185822 |
| 004 | 1 | Human | AF444003 |
| 005 | 1 | Human | AF459438 |
| 006 | 1 | Human | AY204877 |
| 007 | 1 | Human | AY230202 |
| 008 | 1 | Human | D10330 |
| 009 | 1 | Human | D11092 |
| 010 | 1 | Human | D11093 |
| 011 | 1 | Human | DQ459342 |
| 012 | 1 | Human | FJ457024 |
| 013 | 1 | Human | HI577563 |
| 014 | 1 | Human | L08816 |
| 015 | 1 | Human | L25547 |
| 016 | 1 | Human | L25595 |
| 017 | 1 | Human | M73218 |
| 018 | 1 | Human | M94177 |
| 019 | 1 | Human | X98292 |
| 020 | 1 | Human | X99441 |
| 021 | 2 | Human | M74506 |
| 022 | 3 | Human | AB074918 |
| 023 | 3 | Human | AB074920 |
| 024 | 3 | Human | AB089824 |

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| | | | |
|---|---|---|---|
| 025 | 3 | Human | AB091394 |
| 026 | 3 | Human | AB189072 |
| 027 | 3 | Human | AB189073 |
| 028 | 3 | Human | AB189074 |
| 029 | 3 | Human | AB189075 |
| 030 | 3 | Human | AB246676 |
| 031 | 3 | Human | AB248520 |
| 032 | 3 | Human | AB291951 |
| 033 | 3 | Human | AB291952 |
| 034 | 3 | Human | AB291953 |
| 035 | 3 | Human | AB291954 |
| 036 | 3 | Human | AB291955 |
| 037 | 3 | Human | AB291956 |
| 038 | 3 | Human | AB291957 |
| 039 | 3 | Human | AB291958 |
| 040 | 3 | Human | AB291960 |
| 041 | 3 | Human | AB291961 |
| 042 | 3 | Human | AB291962 |
| 043 | 3 | Human | AB291963 |
| 044 | 3 | Human | AB301710 |
| 045 | 3 | Human | AB369687 |
| 046 | 3 | Human | AB369689 |
| 047 | 3 | Human | AB369691 |
| 048 | 3 | Human | AF060668 |
| 049 | 3 | Human | AF060669 |
| 050 | 3 | Human | AP003430 |
| 051 | 3 | Human | EU495148 |

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| | | | |
|---|---|---|---|
| 052 | 3 | Human | FJ653660 |
| 053 | 3 | Human | FJ956757 |
| 054 | 3 | Swine | AB073912 |
| 055 | 3 | Swine | AB248521 |
| 056 | 3 | Swine | AB248522 |
| 057 | 3 | Swine | AB290312 |
| 058 | 3 | Swine | AB290313 |
| 059 | 3 | Swine | AB443623 |
| 060 | 3 | Swine | AB481226 |
| 061 | 3 | Swine | AB481228 |
| 062 | 3 | Swine | AB481229 |
| 063 | 3 | Swine | AF082843 |
| 064 | 3 | Swine | AF455784 |
| 065 | 3 | Swine | AY115488 |
| 066 | 3 | Swine | EU360977 |
| 067 | 3 | Swine | EU375463 |
| 068 | 3 | Swine | EU723512 |
| 069 | 3 | Swine | EU723513 |
| 070 | 3 | Swine | EU723514 |
| 071 | 3 | Swine | EU723515 |
| 072 | 3 | Swine | EU723516 |
| 073 | 3 | Swine | FJ426403 |
| 074 | 3 | Swine | FJ426404 |
| 075 | 3 | Swine | FJ527832 |
| 076 | 3 | Boar | AB189070 |
| 077 | 3 | Boar | AB222182 |
| 078 | 3 | Boar | AB222183 |

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| | | | |
|---|---|---|---|
| 079 | 3 | Boar | AB222184 |
| 080 | 3 | Boar | FJ705359 |
| 081 | 3 | Boar | FJ998008 |
| 082 | 3 | Boar | FJ998015 |
| 083 | 3 | Deer | AB189071 |
| 084 | 3 | Mongoose | AB236320 |
| 085 | 3 | Rabbit | FJ906895 |
| 086 | 3 | Rabbit | FJ906896 |
| 087 | 3 | Rabbit | GU937805 |
| 088 | 4 | Human | AB074915 |
| 089 | 4 | Human | AB074917 |
| 090 | 4 | Human | AB080575 |
| 091 | 4 | Human | AB091395 |
| 092 | 4 | Human | AB097812 |
| 093 | 4 | Human | AB099347 |
| 094 | 4 | Human | AB108537 |
| 095 | 4 | Human | AB161717 |
| 096 | 4 | Human | AB161718 |
| 097 | 4 | Human | AB161719 |
| 098 | 4 | Human | AB193176 |
| 099 | 4 | Human | AB193177 |
| 100 | 4 | Human | AB193178 |
| 101 | 4 | Human | AB197673 |
| 102 | 4 | Human | AB197674 |
| 103 | 4 | Human | AB200239 |
| 104 | 4 | Human | AB220971 |
| 105 | 4 | Human | AB220972 |

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| | | | |
|---|---|---|---|
| 106 | 4 | Human | AB220973 |
| 107 | 4 | Human | AB220974 |
| 108 | 4 | Human | AB220975 |
| 109 | 4 | Human | AB220976 |
| 110 | 4 | Human | AB220977 |
| 111 | 4 | Human | AB220978 |
| 112 | 4 | Human | AB220979 |
| 113 | 4 | Human | AB253420 |
| 114 | 4 | Human | AB291959 |
| 115 | 4 | Human | AB291964 |
| 116 | 4 | Human | AB291965 |
| 117 | 4 | Human | AB291966 |
| 118 | 4 | Human | AB291967 |
| 119 | 4 | Human | AB291968 |
| 120 | 4 | Human | AB369688 |
| 121 | 4 | Human | AB369690 |
| 122 | 4 | Human | AB521805 |
| 123 | 4 | Human | AB521806 |
| 124 | 4 | Human | AB602439 |
| 125 | 4 | Human | AJ272108 |
| 126 | 4 | Human | EF077630 |
| 127 | 4 | Human | FJ763142 |
| 128 | 4 | Human | HM439284 |
| 129 | 4 | Swine | AB097811 |
| 130 | 4 | Swine | AB481227 |
| 131 | 4 | Swine | AY594199 |
| 132 | 4 | Swine | AY723745 |

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| | | | |
|---|---|---|---|
| 133 | 4 | Swine | DQ279091 |
| 134 | 4 | Swine | DQ450072 |
| 135 | 4 | Swine | EF570133 |
| 136 | 4 | Swine | EU366959 |
| 137 | 4 | Swine | EU676172 |
| 138 | 4 | Swine | FJ610232 |
| 139 | 4 | Swine | GU119960 |
| 140 | 4 | Swine | GU119961 |
| 141 | 4 | Swine | GU188851 |
| 142 | 4 | Swine | GU206559 |
| 143 | 4 | Swine | GU361892 |
| 144 | 4 | Swine | HM152568 |
| 145 | 4 | Boar | AB602440 |

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# Tumour susceptibility gene 101 and the vacuolar protein sorting pathway are required for the release of hepatitis E virions

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We have previously demonstrated that an intact PSAP motif in the ORF3 protein is required for the formation and release of membrane-associated hepatitis E virus (HEV) particles with ORF3 proteins on their surface. In this study, we investigated the direct interaction between the ORF3 protein and tumour susceptibility gene 101 (Tsg101), a cellular factor involved in the budding of viruses containing the P(T/S)AP late-domain, in PLC/PRF/5 cells expressing the wild-type or PSAP-mutated ORF3 protein and Tsg101 by co-immunoprecipitation. Tsg101 bound to wild-type ORF3 protein, but not to the PSAP-inactive ORF3 protein. To examine whether HEV utilizes the multivesicular body (MVB) pathway to release the virus particles, we analysed the efficiency of virion release from cells upon introduction of small interfering RNA (siRNA) against Tsg101 or dominant-negative (DN) mutants of Vps4 (Vps4A and Vps4B). The relative levels of virus particles released from cells depleted of Tsg101 decreased to 6.4 % of those transfected with negative control siRNA. Similarly, virion egress was significantly reduced by the overexpression of DN forms (Vps4AEQ or Vps4BEQ). The relative levels of virus particles released from cells expressing Vps4AEQ and Vps4BEQ were 19.2 and 15.6 %, respectively, while the overexpression of wild-type Vps4A and Vps4B did not alter the levels of virus release. These results indicate that the ORF3 protein interacts with Tsg101 through the PSAP motifs in infected cells, and that Tsg101 and the enzymic activities of Vps4A and Vps4B are involved in HEV release, thus suggesting that HEV requires the MVB pathway for egress of virus particles.

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## INTRODUCTION

Hepatitis E virus (HEV), a member of the genus *Hepevirus* in the family *Hepeviridae*, is the causative agent of acute or fulminant hepatitis E, which occurs in many parts of the world, principally as a water-borne infection in developing countries and zoonotically in industrialized countries (Chandra *et al.*, 2008; Colson *et al.*, 2010; Dalton *et al.*, 2008; Purcell & Emerson, 2008; Tei *et al.*, 2003; Yazaki *et al.*, 2003). HEV is a non-enveloped small virus with a diameter of 27–32 nm, present in the bile and faeces of infected hosts, while HEV particles in the circulating blood and culture supernatant are found to be associated with lipids (Takahashi *et al.*, 2008b, 2010; Yamada *et al.*, 2009a). The HEV genome is a positive-sense, ssRNA composed of approximately 7200 nt, which is capped and polyadenylated (Kabrane-Lazizi *et al.*, 1999; Tam *et al.*, 1991). The genome consists of a 5′ UTR, three ORFs, a 3′ UTR and a poly(A) tail at the 3′ terminus (Emerson & Purcell, 2007). ORF1 encodes non-structural proteins including a methyltransferase, papain-like cysteine protease, helicase and RNA-dependent RNA polymerase (Agrawal *et al.*, 2001; Koonin *et al.*, 1992). ORF2 and ORF3 overlap, and the ORF2 and ORF3 proteins are translated from a bicistronic subgenomic RNA 2.2 kb in length (Graff *et al.*, 2006; Ichiyama *et al.*, 2009). The ORF2 protein is the viral capsid protein of 660 aa, while the ORF3 protein is a small protein of only 113 or 114 aa, which has been suggested to act as an adaptor to link the intracellular transduction pathways, reduce the host inflammatory response and protect virus-infected cells (Chandra *et al.*, 2008). Recently, it was demonstrated that the ORF3 protein is important for egress from cultured cells (Emerson *et al.*, 2010; Nagashima *et al.*, 2011; Yamada *et al.*, 2009a).

At least four major genotypes (1–4) have been identified in mammals. While HEV genotypes 1 and 2 have only been found in humans and are associated with epidemics in developing countries, HEV genotypes 3 and 4 are zoonotic

 

and responsible for sporadic cases worldwide (Okamoto, 2007). A number of animal strains of HEV have also been identified from several animal species including chickens, pigs and wild boars (Meng, 2011).

The class E vacuolar protein sorting (Vps) pathway functions at the limiting membrane of multivesicular bodies (MVBs) during lumenal vesicle formation, and is exploited by the late assembly or L-domains of enveloped viruses during budding (Katzmann *et al.*, 2002; Pornillos *et al.*, 2002). Many class E Vps factors are components of three endosomal sorting complexes required for transport (ESCRT-I, -II and -III) (Babst *et al.*, 2002a, b; Katzmann *et al.*, 2001). A current model for viral and MVB vesicle budding invokes the sequential recruitment of ESCRT-I, -II and -III. Thereafter, budding occurs concurrently with the release of ESCRT components from the MVB membrane induced by an AAA-ATPase, Vps4, which is present in humans as two isoforms, Vps4A and Vps4B (Babst *et al.*, 1998; Katzmann *et al.*, 2002). Numerous enveloped RNA viruses such as human immunodeficiency virus type 1 (HIV-1) (Garrus *et al.*, 2001), Ebola virus (Martin-Serrano *et al.*, 2001) and avian sarcoma virus (ASV) (Pincetic *et al.*, 2008) and, more recently, hepatitis C virus (HCV) (Ariumi *et al.*, 2011; Corless *et al.*, 2010; Lai *et al.*, 2010) have been shown to utilize ESCRT complexes during virion morphogenesis. Furthermore, enveloped DNA viruses including hepatitis B virus (HBV) (Lambert *et al.*, 2007; Watanabe *et al.*, 2007) and herpes simplex virus 1 (HSV-1) (Crump *et al.*, 2007) have been reported to exploit the MVB machinery.

Known late domains consist of the amino acid sequence P(T/S)AP, PPxY or YxxL, where 'x' represents any amino acid (Göttlinger *et al.*, 1991; Huang *et al.*, 1995; Wills & Craven, 1991; Yasuda & Hunter, 1998). The PTAP motifs recruit ESCRT-I by binding to Tsg101 (Demirov *et al.*, 2002; Garrus *et al.*, 2001; Martin-Serrano *et al.*, 2001; VerPlank *et al.*, 2001). Tsg101 is a component of ESCRT-I, a cellular complex essential in the Vps pathway, which traffics proteins to the MVB and lysosome (Bishop & Woodman, 2001; Katzmann *et al.*, 2001). Small interfering RNA (siRNA)-mediated Tsg101 depletion potently blocks HIV-1 release (Garrus *et al.*, 2001). Moreover, all late domain (L-domain) types require a subset of class E Vps factors, and their function is blocked by dominant-negative (DN) forms of ESCRT-III components (Martin-Serrano *et al.*, 2003a; Strack *et al.*, 2003; von Schwedler *et al.*, 2003) or Vps4 (Garrus *et al.*, 2001; Martin-Serrano *et al.*, 2003b; Tanzi *et al.*, 2003).

The PSAP motif between amino acid residues 95 and 98 of the ORF3 protein was found to be conserved among all known HEV strains of genotypes 1–4 and avian HEV strains (Nagashima *et al.*, 2011). Surjit *et al.* (2006) reported that cellular Tsg101 binds to the PSAP motif located within the ORF3 protein, and that substitutions in the PSAP sequence abolish the ability of the protein to bind Tsg101. In our previous study, we demonstrated that the PSAP motif(s) in the ORF3 protein is necessary for the egress of HEV particles from infected cells, and proposed that the PSAP motifs in the ORF3 protein play a role as the functional domain required for virion release associated with lipids and the ORF3 protein (Nagashima *et al.*, 2011). However, it remains unclear whether the MVB pathway is involved in HEV virion egress. In this study, we investigated the requirement of Tsg101 and Vps4 during the release of viral particles by using siRNA against Tsg101 and DN mutants of Vps4. The results showed that Tsg101 and the enzymic activities of Vps4A and Vps4B are involved in the release of HEV virions, thus suggesting that HEV utilizes the MVB machinery to exit cells.

## RESULTS

### The ORF3 protein interacts with Tsg101 in cultured cells

In our previous study with the advent of co-immunoprecipitation method, we showed that the exogenously expressed wild-type ORF3 protein interacts with endogenous Tsg101 in PLC/PRF/5 cells, and that this interaction is associated with the PSAP motif(s) in the ORF3 protein (Nagashima *et al.*, 2011). However, endogenous Tsg101 binding to the ORF3 protein was not detected upon immunoprecipitation with an anti-ORF3 mAb, probably because there was insufficient expression of endogenous Tsg101 to permit detection by Western blotting analysis. To confirm the binding of ORF3 protein with Tsg101 *in vivo*, we investigated the direct interaction between the Tsg101 and ORF3 proteins in cells expressing Tsg101 containing a Myc-tag at its N terminus (Tsg101-Myc) and ORF3 protein by co-immunoprecipitation. The PLC/PRF/5 cells were transfected with pBj-Myc-Tsg101 in combination with pCI-HEVORF3/wt or pCI-HEVORF3/mutPLAP/LSAL, or with both empty vectors. As shown in Fig. 1(a), the expression of Tsg101-Myc was detectable by Western blotting analysis using anti-Myc mAb (left panel) and anti-Tsg101 mAb (right panel). Similarly, it was found that wild-type and mutPLAP/LSAL ORF3 proteins were efficiently expressed in the transfected cells (Fig. 1b).

The extracts of the PLC/PRF/5 cells co-transfected with pBj-Myc-Tsg101 in combination with pCI-HEVORF3/wt or pCI-HEVORF3/mutPLAP/LSAL were subjected to immunoprecipitation with either the anti-Myc or anti-ORF3 mAb and then the immunoprecipitates were analysed by Western blotting using either anti-Tsg101 or anti-ORF3 mAb. As shown in Fig. 1(c), the Tsg101-Myc and ORF3 proteins were co-immunoprecipitated with either the anti-Myc or the anti-ORF3 mAb in cell extracts expressing the wild-type ORF3 and Tsg101-Myc proteins. In contrast, the cell extracts expressing the mutated ORF3 protein without intact PSAP motifs (mutPLAP/LSAL) and Tsg101-Myc did not show any co-immunoprecipitates of the Tsg101-Myc and ORF3 proteins. As expected, no immunoprecipitates were observed in extracts of cells transfected with both empty vectors, confirming the specificity of the Western blot analysis. These results confirmed that the ORF3

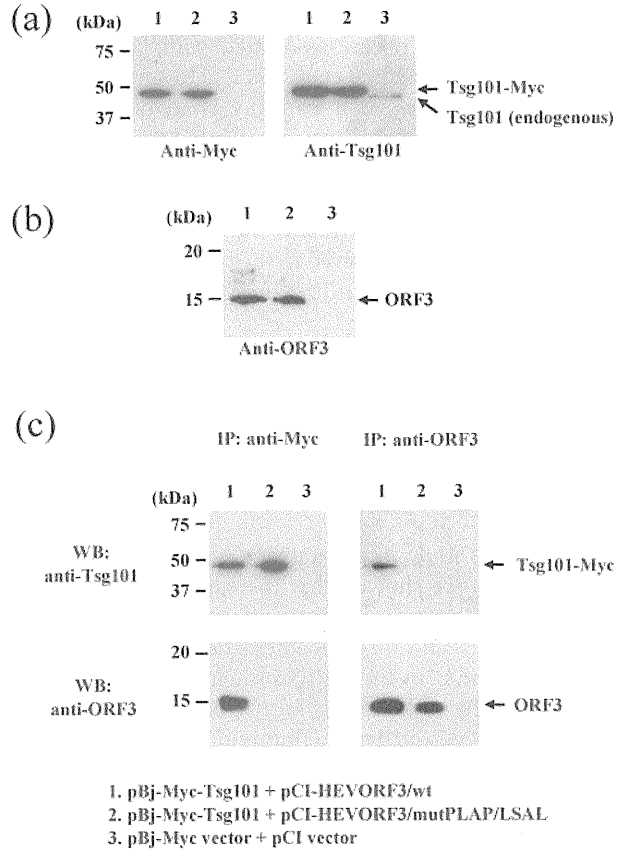


**Fig. 1.** Simultaneous expression of Tsg101-Myc and ORF3 proteins (wild-type or mutPLAP/LSAL) in PLC/PRF/5 cells. PLC/PRF/5 cells were transfected with pBj-Myc-Tsg101 and pCI-HEVORF3/wt (lane 1), pBj-Myc-Tsg101 and pCI-HEVORF3/mutPLAP/LSAL (lane 2) or both empty vectors (pBj-Myc and pCI vectors), which were used as negative controls (lane 3). The cell lysates were subjected to Western blotting analysis with an anti-Myc mAb (left panel) or with an anti-Tsg101 mAb (right panel) (a) or anti-ORF3 mAb (b). (c) Cell extracts were immunoprecipitated with the anti-Myc mAb or anti-ORF3 mAb. The immunoprecipitated proteins were subjected to a Western blotting analysis with the anti-Tsg101 mAb or the anti-ORF3 mAb.

protein interacts with Tsg101 through its PSAP motif *in vivo*.

## Intracellular localization of the ORF3 protein and Tsg101

We subsequently examined the intracellular localization of the ORF3 protein and Tsg101 by immunofluorescence confocal microscopy. First, to confirm the specific detection of the Tsg101-Myc and ORF3 proteins expressed in the transfected cells, PLC/PRF/5 cells were transfected with pBj-Myc-Tsg101, pCI-HEVORF3/wt and pCI-HEVORF3/mutPLAP/LSAL, or with both the pBj-Myc and pCI vectors (Fig. 2a). At 48 h after transfection, cells were stained simultaneously with Alexa Fluor 488-conjugated anti-Myc and Alexa Fluor 594-conjugated anti-ORF3 mAbs. The signal of Myc-tagged Tsg101 stained by anti-Myc was visible only in the cytoplasm of cells transfected with pBj-Myc-Tsg101. Similarly, the signals of wild-type and mutant ORF3 proteins detectable by anti-ORF3 were seen in both cells transfected with pCI-HEVORF3/wt and those with pCI-HEVORF3/mutPLAP/LSAL. In contrast, no specific signals were observed in the cells transfected with both empty vectors. These results indicate that there was specific detection of Tsg101-Myc and ORF3 proteins in the expressed cells by the immunofluorescence assay used.

To examine the intracellular co-localization of the ORF3 protein with Tsg101 through its PSAP motif, PLC/PRF/5 cells were transfected with pBj-Myc-Tsg101, along with pCI-HEVORF3/wt or pCI-HEVORF3/mutPLAP/LSAL. At 48 h post-transfection, the cells were fixed and processed for the immunofluorescence assay (Fig. 2b). At least 20 different cells co-expressing Tsg101-Myc and ORF3 proteins were analysed in two independent experiments. A high degree of co-localization ($92.5 \pm 2.5\%$) of wild-type ORF3 protein and Tsg101 was observed in the cytoplasm. In contrast, the mutPLAP/LSAL ORF3 protein showed essentially no signal of co-localization with Tsg101, despite the fact that the mutated ORF3 protein showed an intracellular localization similar to the wild-type ORF3 protein. These results support the notion that the ORF3 protein interacts with Tsg101 through its PSAP motif in infected cells.

## Functional involvement of Tsg101 in virion release

To examine whether Tsg101 is functionally involved in HEV budding through its interaction with the ORF3 protein, we utilized siRNA to deplete Tsg101 and examined its effect on virion release from infected cells. PLC/PRF/5 cells were treated with 5 nM of siRNA specific for Tsg101 (siTsg101) or negative control siRNA (NC siRNA) 2 days before and 4 days after virus inoculation (Fig. 3a). Two days after the first transfection of siRNA, the treated cells were inoculated with $2.0 \times 10^5$ copies of cell culture-derived HEV. Transfection of siTsg101, but not NC siRNA and buffer only (no siRNA), caused a marked reduction in the levels of endogenous Tsg101 expression in the inoculated cells (Fig. 3b, day 0). In contrast, no discernible alteration in the expression level of cellular $\beta$-actin was observed. The HEV RNA levels in the culture supernatant of cells transfected with NC siRNA or no siRNA increased gradually from 6 days post-inoculation and reached $4.0 \times 10^4$ and $3.8 \times 10^4$ copies $ml^{-1}$ on day 10, respectively (Fig. 3c). In sharp contrast, the HEV RNA level in the culture supernatant of the siTsg101-transfected cells increased only slightly on day 10, reaching $2.5 \times 10^3$ copies $ml^{-1}$. The relative levels of virus particles released from cells depleted of Tsg101 were significantly decreased to 6.4 % of that released from cells transfected with NC siRNA. The depletion of endogenous Tsg101 continued at least until day 10, while $\beta$-actin was detected at equal levels in both cells transfected with siTsg101 and those transfected with NC siRNA or no

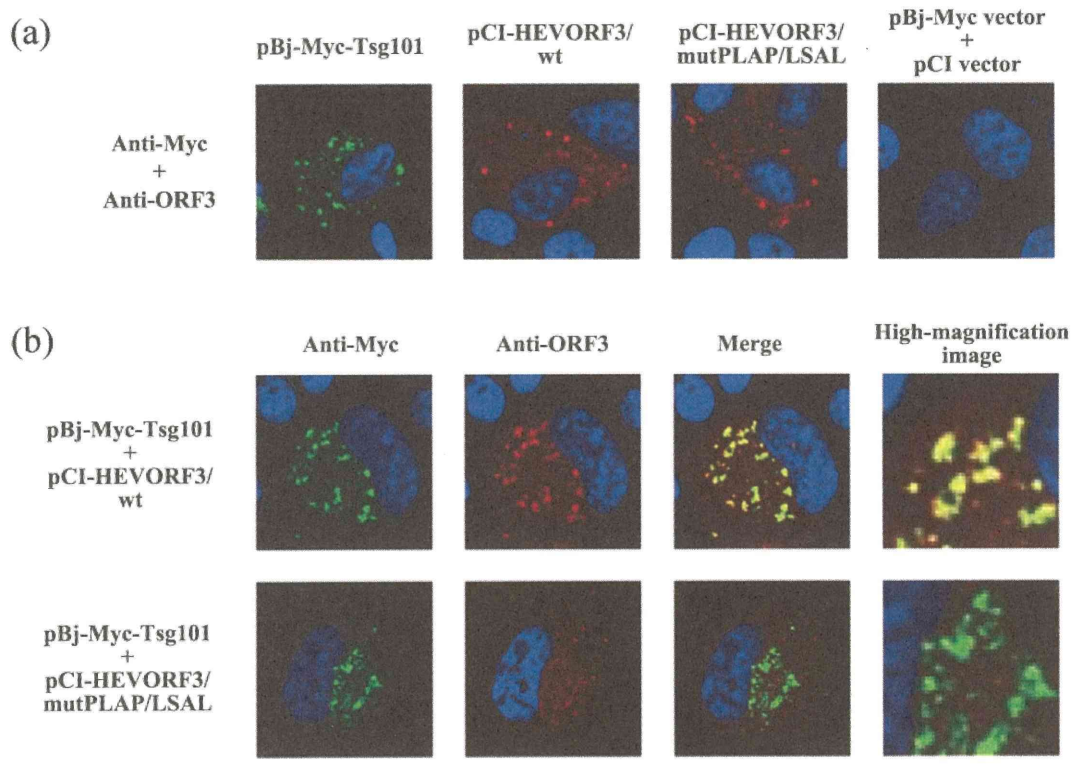


**Fig. 2.** (a) Immunofluorescent staining of the Tsg101-Myc and ORF3 proteins in PLC/PRF/5 cells transfected with pBj-Myc and pCI vectors, or with pBj-Myc-Tsg101, pCI-HEVORF3/wt, or pCI-HEVORF3/mutPLAP/LSAL, by an Alexa Fluor 488-labelled anti-Myc mAb and an Alexa Fluor 594-labelled anti-ORF3 mAb. (b) The intracellular localization of the ORF3 protein and Tsg101 in PLC/PRF/5 cells expressing wild-type or mutant ORF3 proteins, along with Tsg101-Myc. At 48 h after co-transfection with pBj-Myc-Tsg101 and pCI-HEVORF3/wt (upper panel) or pBj-Myc-Tsg101 and pCI-HEVORF3/mutPLAP/LSAL (lower panel), cells were stained with both Alexa Fluor 488-conjugated anti-Myc and Alexa Fluor 594-conjugated anti-ORF3 mAbs. Co-localization is shown in yellow. Nuclei were stained with DAPI. All images are representative of two independent experiments.

siRNA. Next, intracellular viral RNA was serially quantified by two distinct real-time RT-PCR methods with the ORF1 and ORF2/ORF3 probes capable of detecting genomic RNA only (Fig. 3d) and both genomic and subgenomic RNAs (Fig. 3e), respectively. The HEV RNA level in the siTsg101-transfected cells was similar to those in the cells transfected with or without control siRNA at 1, 4 and 8 days post-inoculation, by both of the two distinct quantification methods (Fig. 3d and e), suggesting that the HEV RNA replication was not affected by the siTsg101 transfection. These results clearly indicated that Tsg101 plays a pivotal role specifically in the release of HEV virions. It was also strongly suggested that HEV utilizes the mechanism of cellular MVB sorting, since Tsg101 is a component of the ESCRT-I complex that is involved in MVB sorting.

## The DN forms of Vps4A and Vps4B inhibit the release of virus particles

To examine the involvement of the MVB sorting pathway in the egress of HEV, we analysed the effects of over-expression of two DN forms of Vps4A and Vps4B that play an essential role at the final step of the MVB sorting pathway, on the virus release. PLC/PRF/5 cells were transfected with 0.5 μg of empty vector or expression plasmids for the DN forms of Vps4A and Vps4B, Vps4AEQ and Vps4BEQ (Urata *et al.*, 2006), 2 days before and 2 and 6 days after virus inoculation (Fig. 4a). Two days after the first transfection, the pretreated cells were inoculated with $2.0 \times 10^5$ copies of HEV progeny in the culture supernatant. As shown in Fig. 4(b), the expression of Vps4AEQ and Vps4BEQ was seen in the inoculated cells and continued until day 8. The HEV RNA levels in the culture supernatant of cells transfected with the empty vector or buffer only (no plasmid) increased gradually from 6 days post-inoculation and reached $1.0 \times 10^4$ and $1.1 \times 10^4$ copies $ml^{-1}$ on 8 days, respectively (Fig. 4c). In contrast, the HEV RNA levels in the culture supernatant of cells transfected with Vps4AEQ or Vps4BEQ increased slightly on day 8, with the viral load being $2.2 \times 10^3$ and $1.8 \times 10^3$ copies $ml^{-1}$, respectively (Fig. 4c). The relative levels of virus particles released from cells expressing Vps4AEQ and Vps4BEQ were decreased to 19.2 and 15.6 %, respectively, on day 8 (Fig. 4d). These results suggest that HEV utilizes the Vps pathway for virion egress

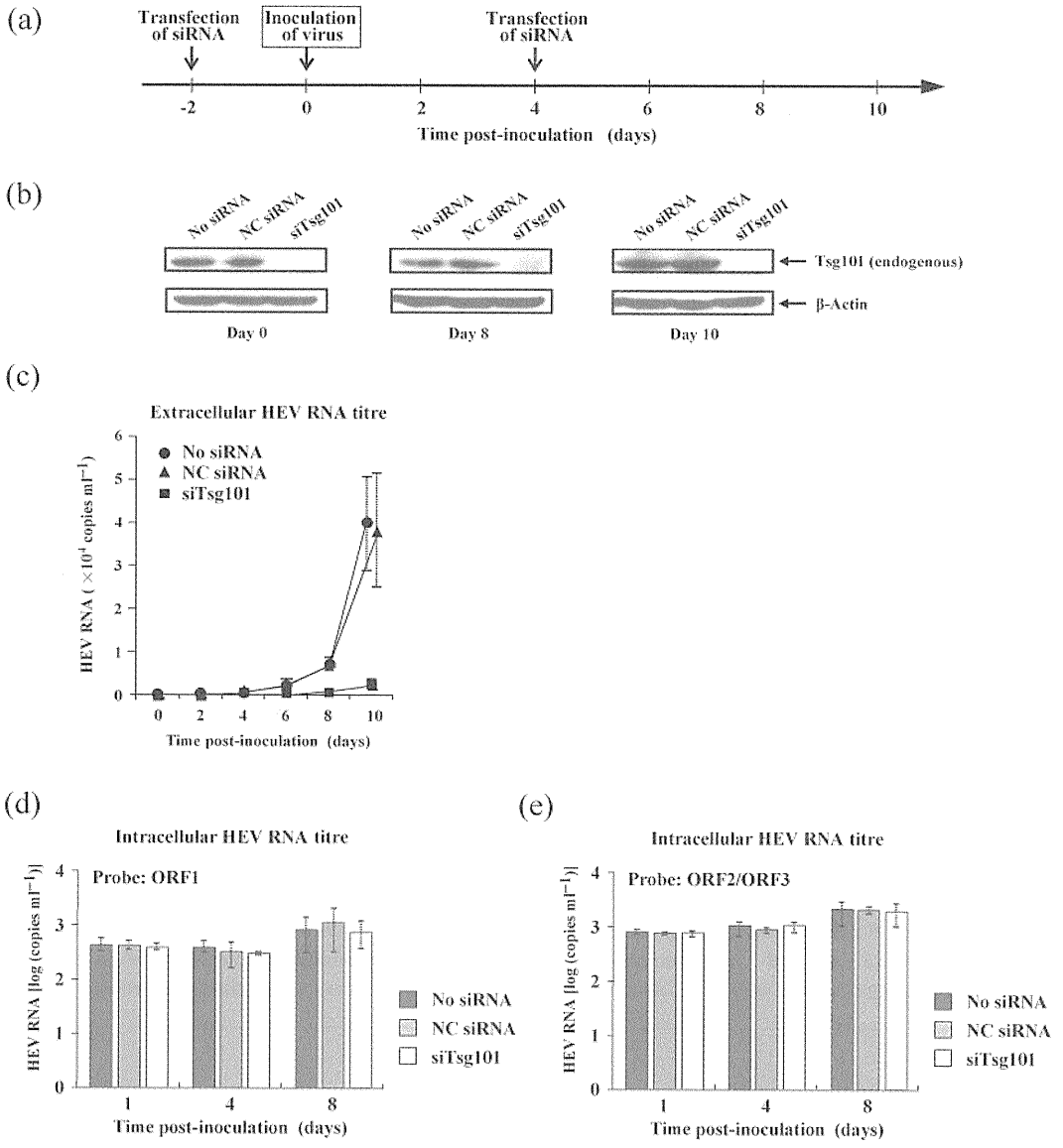


**Fig. 3.** (a) The experimental schedule. PLC/PRF/5 cells were transfected with siRNA 2 days before and 4 days after virus inoculation on day 0. Half of the culture medium was replaced with antibiotic-free growth medium every other day after virus inoculation. (b) The effect of siRNA specific for Tsg101. PLC/PRF/5 cells were treated with siRNA specific for Tsg101 (siTsg101), negative control siRNA (NC siRNA), or buffer only (no siRNA). On the indicated days after inoculation, cells were lysed and then the expression levels of Tsg101 (upper panel) and β-actin (lower panel) were detected by Western blotting analysis using the anti-Tsg101 and anti-β-actin mAbs, respectively. (c) The levels of extracellular HEV RNA in the infected cells treated with siRNA. (d and e) The levels of intracellular HEV RNA in infected cells that were transfected with siRNA. The HEV RNA titre was quantified by real-time RT-PCR with primers targeting the ORF1 region (d) or the ORF2/ORF3 overlapping region (e). All experiments were done in triplicate and the data represent mean ± SD.

and that the enzymic activities of Vps4A and Vps4B are involved in virus release.

## The effects of overexpression of Vps4A and Vps4B on virion egress

To further investigate the functions of Vps4A and Vps4B, PLC/PRF/5 cells were transfected with pVps4A or pVps4B, 2 days before and 2 and 6 days after virus inoculation (Fig. 4a). The expression of Vps4A and Vps4B was observed in inoculated cells and continued at least until day 8 (Fig. 4e). As shown in Fig. 4(f), the overexpression of Vps4A and Vps4B did not promote or reduce virus release. The relative levels of virus particles released from cells expressing Vps4A and Vps4B were estimated to be 104.3 and 103.7 %, respectively, on day 8 (Fig. 4g), suggesting that endogenous Vps4 are

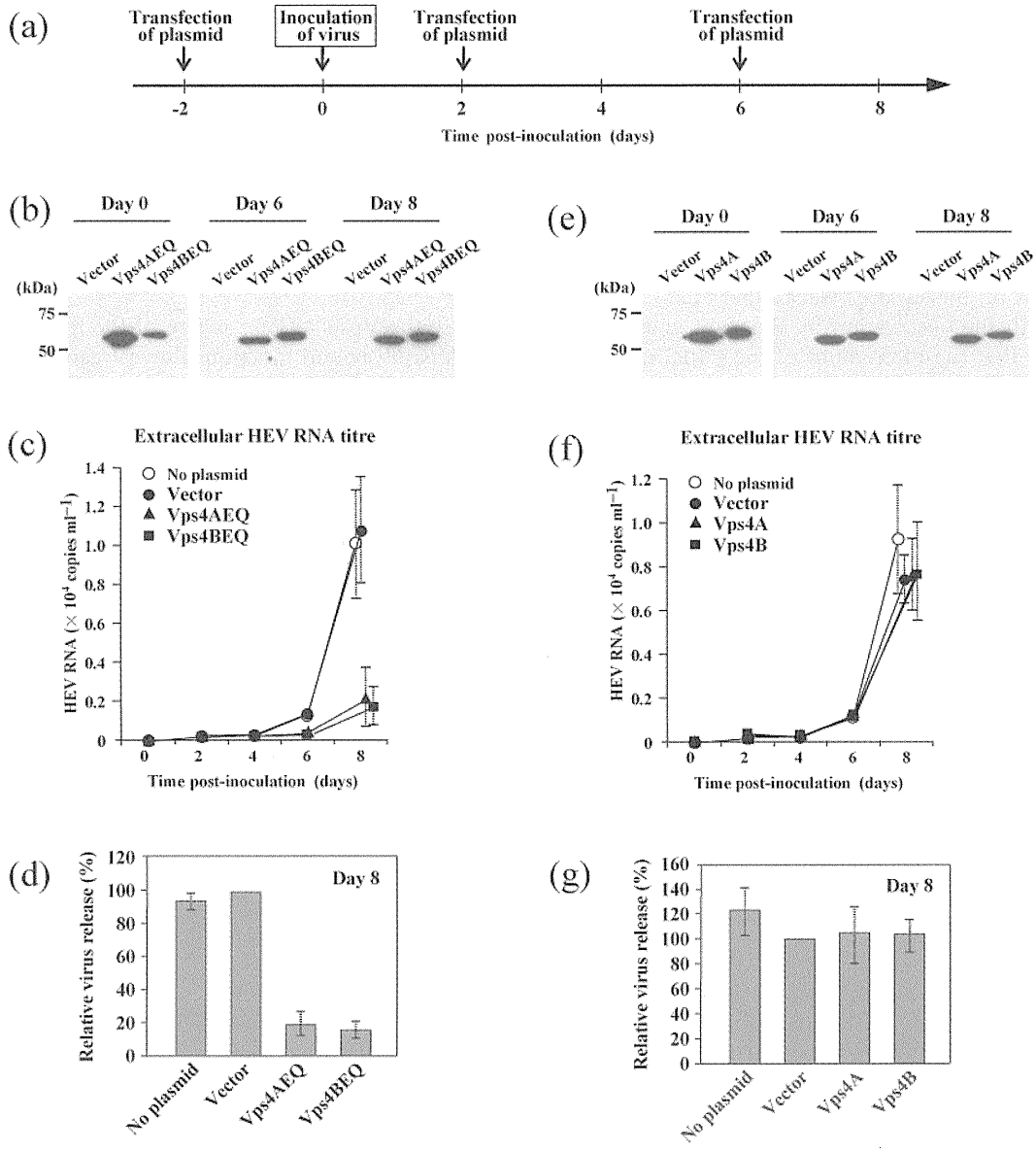


**Fig. 4.** (a) The experimental schedule. PLC/PRF/5 cells were transfected with the expression plasmid 2 days before and 2 and 6 days after virus inoculation on day 0. Half of the culture medium was replaced with growth medium every other day after virus inoculation. (b and e) The expression of DN or wild-type Vps4. PLC/PRF/5 cells were transfected with pVps4AEQ, pVps4BEQ, the empty vector, or buffer only (no plasmid) (b), or pVps4A, pVps4B, the empty vector, or buffer only (e). On the indicated days after inoculation, the cells were lysed and then the Flag-tagged DN forms (b) or wild-type forms (e) of Vps4 were detected by Western blotting analysis using an anti-Flag mAb. (c and f) The levels of extracellular HEV RNA in cells transfected with the DN forms (c) or the wild-type forms (f) of Vps4. The data represent mean ± SD of three independent experiments. (d and g) The efficiency of virus release in the culture supernatant of cells transfected with the DN forms (d) or the wild-type forms (g) of Vps4 at 8 days post-inoculation. The extent of HEV RNA in virus particles released from cells transfected with empty vector was set to 100 %.

sufficient for producing viral particles, and that overexpression does not lead to an increased release of viral particles.

## Co-localization of the ORF3 protein with CD63

To confirm the intracellular co-localization of the ORF3 protein and MVBs, PLC/PRF/5 cells inoculated with wild-type HEV were fixed and stained simultaneously with Alexa Fluor 594-conjugated anti-ORF3 mAb and Alexa Fluor 488-conjugated antibody against CD63, one of MVB marker proteins (Fig. 5). When at least 20 different cells expressing the ORF3 proteins were analysed in two independent experiments, a high degree of co-localization with CD63 (82.5 ± 2.5 %) was observed in the cytoplasm of the infected

cells. In support of the specificity of the immunofluorescence assay, no signal of co-localization was visible in the uninfected cells.

## DISCUSSION

Our previous study showed the requirement of an intact PSAP motif in the ORF3 protein for the formation and release of membrane-associated HEV particles possessing ORF3 proteins on the surface (Nagashima *et al.*, 2011), in agreement with the observation by Emerson *et al.* (2010) who reported that the PxxP motif of the ORF3 protein is important for virus egress and infection. The role of host protein(s) in ORF3-mediated HEV release remained unknown, although the requirement of the PSAP motif suggested a possible role for Tsg101, which was shown to bind the ORF3 protein (Nagashima *et al.*, 2011; Surjit *et al.*, 2006). In the present study, we confirmed that the ORF3 protein interacts with Tsg101 through a PSAP motif in cultured cells and demonstrated that depletion of endogenous Tsg101 by siRNA led to a significant reduction of HEV release in cultured cells. Furthermore, we demonstrated for the first time that HEV utilizes the Vps machinery to support the release of membrane-associated particles with the ORF3 protein on the surface in a Tsg101-dependent manner. These results indicate that the PSAP motif in the ORF3 protein acts as an L-domain for the release of membrane-associated HEV particles from infected cells.

Efficient release of an enveloped virus requires viral L-domains, including P(T/S)AP, YxxL and PPxY amino acid motifs, which are found in the structural proteins of enveloped viruses and hijack host proteins in the Vps pathway (Göttlinger *et al.*, 1991; Huang *et al.*, 1995; Wills & Craven, 1991). This pathway gives rise to MVBs, which are topologically identical to virus budding (Chen & Lamb, 2008). In the present study, we showed evidence of binding between the PSAP motif of the ORF3 protein and Tsg101, which was found to be essential for the release of HEV virions, since interference with Tsg101 expression by siRNA inhibited the release of HEV (Fig. 3). This finding indicates an important role for Tsg101 in the release of HEV particles, corroborating the role attributed to Tsg101 in the budding of other known enveloped viruses such as retroviruses (Garrus *et al.*, 2001), although HEV is a non-enveloped virus.

Since the Vps4 ATPase is one of the final effectors in the Vps pathway and functions downstream of Tsg101, it was hypothesized that DN mutants of Vps4 might disrupt virion release. In fact, DN mutants of Vps4 have been shown to inhibit the budding of infectious HIV-1 particles by more than 200-fold in co-transfection experiments, whereas overexpression of wild-type Vps4 inhibited the budding of HIV-1 by only two- to fourfold (Garrus *et al.*, 2001). Our present study indicated that overexpression of Vps4AEQ or Vps4BEQ inhibits the release of HEV, with the relative levels of virus particles released from cells expressing Vps4AEQ and Vps4BEQ being 19.2 and 15.6 % compared with wild-type cells, respectively (Fig. 4). The reduction in the release of HEV particles in the presence of DN Vps4A or Vps4B appears to be due to a specific block in the formation of membrane-associated virions. It is likely that Vps4 is required for the final step in the formation of membrane-associated HEV particles.

Many enveloped viruses complete their replication cycle by budding from the plasma membrane (Demirov *et al.*, 2002; Hartlieb & Weissenhorn, 2006; Jayakar *et al.*, 2004). RNA viruses, including HIV and the Ebola virus, utilize the cellular ESCRT machinery to facilitate their escape from host cells by redirecting ESCRT complexes to the cell surface, where they appear to drive the budding and fission of the viral particles (Demirov *et al.*, 2002; Garrus *et al.*,

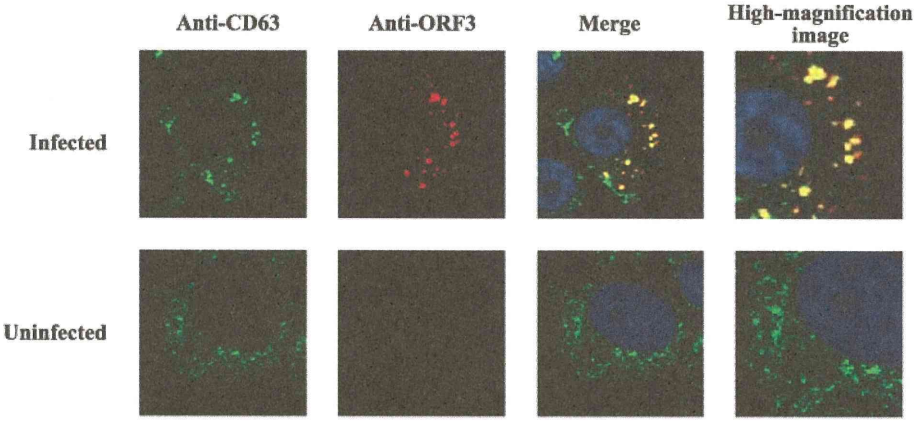


**Fig. 5.** Co-localization of the ORF3 protein and CD63 in PLC/PRF/5 cells infected with cell culture-produced wild-type HEV. At 20 days post-inoculation, the infected cells and uninfected cells as a control were stained with both Alexa Fluor 488-conjugated anti-CD63 polyclonal antibody and Alexa Fluor 594-conjugated anti-ORF3 mAb. Co-localization is shown in yellow. Nuclei were stained with DAPI. All images are representative of two independent experiments.

2001; Martin-Serrano *et al.*, 2003a). Recently, Lai *et al.* (2010) reported that HCV egress requires the motility of early to late endosomes, which is microtubule-dependent, and postulated that following the assembly of virus particles in juxtaposition to lipid droplets, the HCV particles are transported through early to late endosomes to the plasma membrane, where the membrane of late endosomes is fused with the plasma membrane to release virions into the extracellular milieu. In most herpesviruses, nucleocapsids with tegument proteins are known to bud into intracellular vesicles formed in the infected cells, which are thought to be derived from the Golgi apparatus, *trans*-Golgi network (TGN) or endosomes (Crump *et al.*, 2007; Fraile-Ramos *et al.*, 2007), and the virions are then released into the extracellular environment together with small vesicles by the exosomal secretion pathway.

It remains unknown whether the membrane-associated HEV particles are generated intracellularly or at the cell surface (Ahmad *et al.*, 2011). HEV particles with lipid membranes and the ORF3 protein on their surface were found abundantly in the lysates of cells infected with wild-type HEV (S. Nagashima and H. Okamoto, unpublished observations), and the ORF3 protein and Tsg101 were co-localized in the cytoplasm (Fig. 2), thus suggesting that mature membrane-associated HEV particles are generated before their release from the surface of infected cells. The immunofluorescence assay using anti-ORF3 mAb and antibody against CD63, another MVB marker protein, revealed that the ORF3 protein is co-localized with CD63 in the HEV-infected cells (Fig. 5). Taken altogether, these findings indicate that it is likely that HEV utilizes the cellular ESCRT machinery in the cytoplasm, but not at the cell surface, to induce the release from infected cells. Further studies are needed to clarify whether membrane-associated HEV particles really bud into the intracellular vesicles formed in infected cells, and whether they are derived from the Golgi apparatus, TGN or endosomes, and also whether the mature virions are released into the extracellular environment by the exosomal secretion pathway, similar to known enveloped viruses such as HCV (Crump *et al.*, 2007; Fraile-Ramos *et al.*, 2007; Lai *et al.*, 2010).

A conserved enveloped virus release mechanism is likely to be an excellent target for antiviral drugs. Recently, tetherin was identified as a cellular factor that inhibits the release of HIV-1 from infected cells (Neil *et al.*, 2008). It appears to prevent HIV-1 release by retaining fully formed progeny virions on the surfaces of infected cells (Neil *et al.*, 2008; Van Damme *et al.*, 2008). Subsequent studies have shown that human tetherin/BST-2 also inhibits the release of filoviruses, arenaviruses and herpesviruses (Jouvenet *et al.*, 2009; Kaletsky *et al.*, 2009; Mansouri *et al.*, 2009; Sakuma *et al.*, 2009). With regard to HEV, although it has been reported that monotherapy using interferon or ribavirin inhibits the viral replication in immunocompromised patients with chronic HEV infection following solid-organ transplantation (Haagsma *et al.*, 2010; Kamar *et al.*, 2010), virtually no approved antiviral drugs are available to prevent or treat HEV-associated diseases. It may therefore be interesting to analyse whether tetherin/BST-2 inhibit the virion release of HEV. A better understanding of the mechanisms whereby viruses recruit cellular factors should aid greatly in the development of this class of therapeutics.

In conclusion, the present study revealed that HEV recruits Tsg101 via its PSAP motif in the ORF3 protein and requires the L-domain function for virion release from infected cells, and that the enzymic activity of Vps4 is involved in virus release. This suggests that, although HEV is known to be a non-enveloped virus, it requires the MVB pathway for its release from infected cells. To support this hypothesis, immune electron microscopy or fluorescence microscopy showing that HEV is indeed in MVB should be performed in future studies.

## METHODS

**Cell culture.** PLC/PRF/5 cells (ATCC CRL-8024) were grown in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10 % (v/v) heat-inactivated FBS (Hana-Nesco Bio), 100 U penicillin G $ml^{-1}$, 100 μg streptomycin $ml^{-1}$ and 2.5 μg amphotericin B $ml^{-1}$ (growth medium), at 37 °C in a humidified 5 % $CO_2$ atmosphere, as described previously (Tanaka *et al.*, 2007).

**Viruses.** HEV progenies ($1.3 \times 10^8$ copies $ml^{-1}$) in the culture supernatant of PLC/PRF/5 cells transfected with RNA transcripts of an infectious cDNA clone of a genotype 3 HEV (pJE03-1760F/wt: DDBJ/EMBL/GenBank accession no. AB437316) (Yamada *et al.*, 2009b) were used for virus inoculation at $10^6$ copies per well (six-well plate). The pJE03-1760F/wt was obtained from a faecal specimen containing a high load of wild-type HEV (strain JE03-1760F: $2.0 \times 10^7$ copies $ml^{-1}$), which can replicate efficiently in PLC/PRF/5 cells (Tanaka *et al.*, 2007).

**Plasmids.** The expression plasmids for the ORF3 protein, pCI-HEVORF3/wt and pCI-HEVORF3/mutPLAP/LSAL, whose two PSAP motifs of the ORF3 protein were changed to PLAP (aa 86–89) and LSAL (aa 95–98), respectively, were reported previously (Nagashima *et al.*, 2011; Takahashi *et al.*, 2008b): all known HEV strains harbour PSAP motif at aa 95–98, while some of the HEV genotype 3 strains, including JE03-1760F, possess an additional PSAP motif at aa 86–89. Plasmids that express Myc-tagged Tsg101 (pBj-Myc-Tsg101) and Flag-tagged Vps4 [pVps4A, pVps4B, and DN mutants of pVps4A and pVsp4B, termed pVps4AEQ (E228Q) and pVps4BEQ (E235Q), respectively] were also described previously (Urata *et al.*, 2006, 2007; Yasuda *et al.*, 2003).

**Protein expression and co-immunoprecipitation.** PLC/PRF/5 cells were transfected with pBj-Myc-Tsg101 and pCI-HEVORF3/wt or pCI-HEVORF3/mutPLAP/LSAL using the TransIT-LT1 reagent (Mirus Bio) according to the manufacturer's recommendations. Both empty vectors [pBj-Myc vector (Yasuda *et al.*, 2003) and pCI vector (Takahashi *et al.*, 2008b)] were used as negative controls. At 48 h after transfection, co-immunoprecipitaion assays were performed using an immunoprecipitation kit (Protein G; Roche) according to the manufacturer's recommendations. After clarification by centrifugation, cell lysates were used for immunoprecipitation with an anti-Myc mAb (9E10; Santa Cruz Biotechnology) or anti-ORF3 mAb (TA0536) (Takahashi *et al.*, 2008b). The co-immunoprecipitated proteins (Tsg101-Myc and ORF3/wt or ORF3/mutPLAP/LSAL) were separated by 10 or 15 % SDS-PAGE, followed by Western blotting analysis with an anti-Tsg101 mAb (C-2; Santa Cruz