

1 **The ATP synthase subunit  $\beta$  (ATP5B) is an entry factor for the**  
2 **hepatitis E virus**

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19

20 **Abstract**

21 Hepatitis E occurs sporadically and as outbreaks due to contamination of drinking  
22 water. The causative agent, hepatitis E virus (HEV) is a hepatotropic non-enveloped  
23 RNA virus, which grows poorly *in vitro*. Consequently, many aspects of HEV biology  
24 are poorly characterized, including its cellular receptor and entry mechanism(s).  
25 Previous studies from our laboratory have shown that heparan sulfate proteoglycans  
26 (HSPGs) act as attachment factors for the virus. In the absence of purified high titer  
27 infectious virus, we have used hepatitis E virus-like particles (HEV-LPs) expressed  
28 and purified from *E. coli* to identify HEV entry factor(s) on liver cells in culture. Using  
29 a pull down and mass spectrometric approach, we identified the ATP synthase  
30 subunit  $\beta$  (ATP5B) to bind the HEV capsid protein. Its role in the entry of HEV was  
31 then validated using antibody and siRNA mediated approaches, and infectious HEV  
32 from the stools of a hepatitis E patient. Though ATP synthase is largely a  
33 mitochondrial protein, the cell surface expressed form of ATP5B is implicated in other  
34 viral infections.

35

## 36 INTRODUCTION

37 Hepatitis E virus (HEV) causes an acute and generally self-limited viral hepatitis with  
38 a mortality rate of 0.5-4% in the general population, which can be as high as 20-30%  
39 in infected pregnant women (Khuroo, 2011). The virus is enterically transmitted and  
40 several large outbreaks have occurred in Asia and Africa due to contamination of  
41 drinking water with fecal matter. In developed countries, HEV is transmitted primarily  
42 through the consumption of raw or undercooked meat from infected animals, with  
43 pigs being an important reservoir (Meng, 2011; Sonoda et al., 2004; Yugo & Meng,  
44 2013).

45 The HEV is classified as a Hepevirus in the family *Hepeviridae* (Smith et al., 2014). It  
46 is a 27-34 nm non-enveloped virus with a ~7.2 kb positive sense polyadenylated and  
47 capped RNA genome (Emerson & Purcell, 2006). Short untranslated regions (UTRs)  
48 flank the coding region of the genome, which includes three open reading frames  
49 (ORFs) called *orf1*, *orf2* and *orf3* (Emerson & Purcell, 2006). Of these, *orf2* encodes  
50 the viral capsid protein (Jameel et al., 1996). Efficient cell culture systems and  
51 suitable small animal models are not available for the propagation of HEV, due to  
52 which details of HEV biology are limited to the role of individual ORFs and the  
53 proteins encoded by these through subgenomic or replicon expression strategies.  
54 The receptor(s) for HEV entry have also not been identified thus far. *In vitro*  
55 transcribed and capped viral genomic RNA is infectious for some cultured cells and  
56 non-human primates following transfection and intrahepatic injection, respectively.  
57 However, the infectivity and cell-to cell-spread of the virions thus generated is poor  
58 (Tanaka et al., 2007).

59 The structure of HEV has been studied by expressing the ORF2 capsid protein in  
60 various surrogate systems, such as insect cells, mammalian cells and *E. coli* (Zheng  
61 et al., 2010; Zhang et al., 2001). Of these, p239 (aa 368-606), which contains the  
62 surface localized protruding domain, when expressed in *E. coli*, folds into 23 nm  
63 virus-like particles with a T=1 symmetry that antigenically and structurally resemble  
64 HEV (Ahmad et al., 2011). Further, in human clinical trials p239 has shown efficacy  
65 as a preventive vaccine (HEV 239) against hepatitis E (Li et al., 2005; Zhu et al.,  
66 2010).

67 The initial attachment of many viruses to host cells takes place via cell surface  
68 glycolipids or glycoproteins (de Haan et al., 2005; Vlasak et al., 2005). This is a  
69 relatively non-specific electrostatic interaction that allows viral particles to be  
70 concentrated on the cell surface. Following initial attachment, most viruses bind to  
71 high affinity receptor(s) leading to entry and productive infection (Grove & Marsh,  
72 2011). The nature of the molecules(s) utilized by a virus to bind and enter cells  
73 determines its tropism, internalization, uncoating and trafficking to sites of  
74 intracellular replication. Some viruses bind single receptors, such as CD155 for the  
75 poliovirus (Mendelsohn et al., 1989) or low density lipoprotein receptor (LDLR) for  
76 human rhinovirus 2 (Hofer et al., 1994). Other viruses bind multiple molecules on the  
77 host cell, which usually occurs in temporally organized steps. For example, the SARS  
78 coronavirus can bind either angiotensin converting enzyme (ACE) or liver-SIGN (L-  
79 SIGN) to gain entry into cells (Li et al., 2003; Jeffers et al., 2004). The human  
80 immunodeficiency virus (HIV) binds two distinct molecules on the cell surface, CD4  
81 as the primary receptor (Dalglish et al., 1984; Klatzmann et al., 1984) followed by  
82 either CCR5 or CXCR4 as the co-receptor (Choe et al., 1996; Deng et al., 1996),  
83 both interactions being essential for productive infection of target cells by HIV. The

84 hepatitis C virus (HCV) uses multiple entry factors - Scavenger receptor-B1 (SR-B1)  
85 (Scarselli et al., 2002), CD81 (Pileri et al., 1998), tight junction proteins Claudin-1  
86 (CLDN1) (Evans et al., 2007), Occludin (OCLN) (Ploss et al., 2009) and other  
87 molecules like EGFR and EphA2 (Lupberger et al., 2011), Transferrin receptor  
88 (Martin and Uprichard, 2013) and Niemann-Pick C1-like 1 cholesterol absorption  
89 receptor (NPC1L1) (Sainz et al., 2012). Enveloped viruses bind target cells through  
90 their surface proteins embedded in the lipid envelopes, while non-enveloped viruses  
91 bind via their capsid protein(s). In either case, the binding of viruses to receptors  
92 triggers signalling leading to activation of endocytosis or modulation of actin  
93 dynamics for lateral movement and clustering of the virus on the plasma membrane  
94 prior to entry (Lehmann et al., 2005; Burckhardt & Greber, 2009). It can also directly  
95 trigger conformational changes in the virus structure to an unstable intermediate that  
96 releases the genome. Following receptor engagement, some enveloped viruses (e.g.  
97 HIV) undergo fusion and non-enveloped viruses (e.g. poliovirus) undergo uncoating  
98 at the cell surface (Kelian & Rey, 2013; Sieczkarski & Whittaker, 2005; De Sena &  
99 Mandel, 1977). Most viruses, however, are internalized by endocytosis into  
100 endosomal compartments and reach their site of replication by escaping from  
101 endosomes. The trigger for this process can be low pH or proteolysis of the capsid by  
102 proteases in early or maturing endosomes (Marsh & Helenius, 2006). Examples of  
103 this include acidification of the influenza virus core due to H<sup>+</sup> translocation through its  
104 M2 protein (Wharton et al., 1994), and cleavage of the Ebola virus glycoprotein by  
105 Cathepsin to reveal determinants that bind the endosomal receptor NPC-1, allowing  
106 translocation to the cytosol (Chandran et al., 2005; Cote et al., 2011; Carette et al.,  
107 2011).

108 Previous studies from our laboratory have shown that HEV binds liver cells via  
109 heparin sulphate proteoglycans (HSPGs) present on cell surface Syndecans (Kalia et  
110 al., 2009). We have recently used the HEV p239 VLP expressed in *E. coli* and virus  
111 infection to show that HEV uses a Clathrin, Dynamin-2 and membrane cholesterol  
112 dependant entry pathway in liver cells (Holla et al., 2015). Here, we use the same  
113 VLPs to pull down proteins in purified membrane fractions from permissive (Huh-7)  
114 and non-permissive (HEK293) cell lines. This approach and further validation using  
115 antibody and siRNA-mediated inhibition, fluorescent imaging and virus infection  
116 identified that ATP synthase subunit  $\beta$  (ATP5B) is involved in the entry of HEV. This  
117 is the first study to identify an entry factor for HEV.

118

## 119 MATERIALS AND METHODS

120 **Cells and reagents.** Huh-7, PLC/PRF/5, HeLa, HEK293 and A549 cells were grown  
121 in DMEM supplemented with 10% Fetal Calf Serum (FCS), 100 U/mL Penicillin and  
122 100  $\mu$ g/mL Streptomycin (all from Gibco, Life Technologies). PCR primers were  
123 designed manually and obtained from Sigma Aldrich (Bengaluru, India). Phusion Hi-  
124 Fidelity DNA polymerase was from Finnzymes (Thermo Fischer Scientific, Inc.),  
125 restriction enzymes and calf intestinal phosphatase (CIP) were from New England  
126 Biolabs Inc., and DNA Ligase was from Promega Corporation. The Nickel-NTA  
127 Superflow beads were obtained from Qiagen, Germany. The cell surface protein  
128 isolation kit and Zeba Desalting columns were purchased from Thermo Scientific,  
129 USA, the protease inhibitor cocktail was from Roche, and mass spectrometry grade  
130 Trypsin Gold was from Promega Corporation. On Target PLUS Smart Pool siRNAs  
131 against ATP5B were purchased from Dharmacon Inc. CTxB-biotin was from Sigma

132 Aldrich (Bengaluru, India). Antibodies were obtained from the following sources: HEV  
133 capsid protein (Anti-HEV capsid antibody, clone 1E6) from LS Bio (USA); ATP5B (N-  
134 terminal region) from Sigma-Aldrich; Cytochrome C, Calnexin, Actin, GAPDH,  
135 MAPK/ERK and EGFR from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA);  
136 Alexa Fluor 488 and Alexa Fluor 568 coupled anti-rabbit and anti-mouse antibodies,  
137 and HRP-conjugated secondary anti-goat, anti-rabbit and anti-mouse IgG from  
138 Calbiochem and Millipore (MA, USA).

139 **Transfections.** Plasmid transfections were done with jetPRIME (Polypus  
140 Transfection) according to manufacturer's instructions. For siRNA transfections, 0.25  
141  $\times 10^6$  cells (Huh-7 or HEK293) were seeded in each well of a 6-well plate and grown  
142 without antibiotics. After reaching 70% confluence, cells were transfected with 50 nM  
143 siRNA against ATP5B or with a non-specific scrambled siRNA as control, incubated  
144 for 4 hours in a serum-free medium followed by complete medium without antibiotics  
145 for 36 hours. At this time, cells were transfected again with siRNA (to ensure  
146 maximum knockdown) and harvested after 72 hours of the first transfection. The cells  
147 were then used for either western blotting or flow cytometry.

#### 148 **Cloning, expression, purification, characterization and labelling of HEV-LP.**

149 Cloning of the HEV-LP was done as described previously (Holla et al., 2015). Briefly,  
150 the *orf2* region corresponding to amino acids 368-606 (nucleotides 1104-1821) were  
151 PCR amplified from the pSK-HEV2 plasmid (Sar-55 strain, genotype 1) with primers  
152 containing NcoI and XhoI sites, and cloned into the pET28b vector. The positive  
153 clones were transformed into the *E. coli* expression strain BL21(DE3) and the VLP  
154 monomer was expressed as a C-terminally 6x Histidine-tagged protein. For  
155 expression, 250 ml bacterial culture was induced with 1 mM isopropyl-D-

156 thiogalactosidase (Sigma-Aldrich) for 4 hours at 37°C. The cells were harvested by  
157 centrifugation and broken by brief sonication in lysis buffer (50 mM Tris-Cl, pH 8.0, 5  
158 mM EDTA, 10mM NaCl, 0.5% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride  
159 and 0.1% NaN<sub>3</sub>). The cell lysate was then treated with 10 mM MgSO<sub>4</sub> for 10 min at  
160 room temperature to chelate EDTA followed by 0.1mg/ml lysozyme for 20 min at  
161 37°C. Inclusion bodies were pelleted by centrifugation at 8,000 rpm (Sorvall SLA 300  
162 rotor) for 15 min at 4°C, resuspended in 100 mM Tris-Cl, pH 8.0 with 100 mM glycine  
163 and sonicated for 2 min. The resuspended inclusion bodies were added dropwise to  
164 the dispersion medium (100 mM Tris pH 8.0, 50 mM glycine, 8 M urea, 5 mM  
165 reduced glutathione and 0.5 mM oxidized glutathione, and left overnight on a rocker.  
166 This preparation was then bound to Ni-NTA bead (Qiagen, Germany) for 1 hour at  
167 room temperature on a rotaspin machine. The beads were washed with binding  
168 buffer containing 20 mM imidazole and bound proteins were eluted in binding buffer  
169 containing 200 mM imidazole. The eluted protein was concentrated using a 10 kDa  
170 Amicon filter (Millipore), subjected to refolding by rapidly diluting 40-fold in a refolding  
171 buffer containing 1 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized  
172 glutathione, 0.5 mM Arginine and 20 mM ethanolamine, pH 8.0, for 36 hr at 4°C. The  
173 refolded protein was concentrated again using a 10 kDa Amicon filter and dialyzed  
174 against PBS. Electron microscopy and FITC labeling of the HEV-LP have been  
175 described elsewhere (Holla et al., 2015).

176 **Surface protein isolation.** Cell surface proteins were isolated with two different  
177 methods. The first used the Total Cell Surface protein isolation Kit (Thermo Scientific)  
178 with some modifications. Briefly, cells were grown in 100 mm dishes up to 80%  
179 confluence, washed and treated with Sulfo-NHS-SS-Biotin in PBS for 30 min on ice.  
180 After washing, the cells were treated with Quenching Solution, pelleted and lysed in



181 PBS containing the protease inhibitor cocktail by sonication at 25 amp pulse for 5 sec  
182 (and a 10 sec off interval) for a total of 3 min on ice. The lysate was incubated with  
183 NeutraAvidin agarose beads for 2 hr at 4°C with end-to-end mixing. The beads were  
184 washed with PBS and bound biotinylated proteins were eluted in PBS containing 50  
185 mM DTT, which was subsequently removed by dialysis against PBS. In the second  
186 approach, membrane fractions were isolated by ultracentrifugation. Cells in 100 mm  
187 dishes were washed with PBS, scraped and lysed in PBS containing protease  
188 inhibitor cocktail as above. The cell debris and nuclei were removed by centrifugation  
189 at 600 x g for 10 min at 4°C, and the membrane fraction was pelleted down in a  
190 SW41Ti rotor (Beckman) at 29,000 rpm for 1 hr at 4°C. This membrane pellet was  
191 washed once with PBS containing protease inhibitor cocktail by ultracentrifugation as  
192 above and resuspended in 400 µl PBS. This was layered over a sucrose step  
193 gradient made up of 900 µl each of 45%, 35%, 20% and 5% sucrose (w/v) up to a  
194 total volume of 4 ml. The gradient was centrifuged at 42,000 rpm in a SW55-Ti rotor  
195 for 16 hr at 4°C. From this, nine fractions of 330 µl were collected from the top (F1-  
196 F9) and 40 µl of each fraction was resolved on a SDS-12% polyacrylamide gel,  
197 transferred to nitrocellulose membrane and western blotting was done for ATP5B,  
198 Calnexin, Cytochrome c and EGFR.

199 **Mass spectrometric identification of membrane proteins.** To identify HEV-LP  
200 interacting membrane proteins, the fractions from Huh-7 and HEK293 cell lines were  
201 pre-cleared with Ni-NTA beads for 2 hr before incubating with purified HEV-LP  
202 overnight at 4°C on a rotator. The mixture was then added to Ni-NTA beads and  
203 incubated at room temperature for 1 hr. The beads were washed and proteins were  
204 eluted by boiling in 1x SDS loading dye at 95°C for 10 min. Proteins were separated  
205 by SDS-PAGE and stained with Coomassie Blue. The bands were excised, cut into

206 small pieces and in-gel tryptic digestion was carried out as described (Shevchenko et  
207 al., 2006). Briefly, gel pieces were destained in 100  $\mu$ l of destaining solution (25 mM  
208  $\text{NH}_4\text{HCO}_3$  and 50%  $\text{CH}_3\text{CN}$ ) by rinsing 3-4 times, centrifuging at 1000 rpm for 1 min  
209 and discarding the supernatant till the gel pieces were completely destained. These  
210 were then dried in a vacuum concentrator. Trypsin solution (13 ng/ $\mu$ l, in 25 mM  
211  $\text{NH}_4\text{HCO}_3$ ) was added to the dried gel pieces and incubated at 37°C overnight,  
212 following which the sample was centrifuged at 1000 rpm for 1 min and digested  
213 peptides were extracted in 20  $\mu$ l of extraction solution (75%  $\text{CH}_3\text{CN}$  and 5%  
214 Trifluoroacetic acid) by vortexing for 30 min. The supernatant was collected, the  
215 extraction procedure was repeated 3-4 times, and the pooled extracts dried in a  
216 vacuum concentrator before submitting for mass spectrometric analysis. For the in-  
217 sol tryptic digestion, total cell surface fractions from Huh-7 and HEK293 cells were  
218 isolated according to the kit protocol. The final eluate, which contained 50 mM DTT,  
219 was mixed with 6 volumes of UA buffer (8 M urea in 0.1 M Tris/HCl pH 8.5) and  
220 added onto a 30 kDa cut-off Nanosep centrifugal filter (Pall Corporation) and  
221 centrifuged at 14,000 x g for 15 min. Three volumes of IAA solution (0.05 M  
222 iodoacetamide in UA buffer) were added to the filter for 20 min, followed by four  
223 washes with UA buffer. The filter was then equilibrated for tryptic digestion by  
224 incubating with ABC solution (0.05 M  $\text{NH}_4\text{HCO}_3$ ) for 20 min, and centrifuging at  
225 14,000 x g. This step was repeated twice before adding trypsin diluted in ABC buffer  
226 (trypsin to protein ratio 1:100) for 8 hr at 37°C. The digested peptides were eluted in  
227 a new collection tube, acidified with trifluoroacetic acid (TFA) and subjected to mass  
228 spectrometry.

229 **Immunofluorescent staining and confocal microscopy.** For all microscopy  
230 experiments, cells were grown on coverslips in 12-well plates. To stain intracellular

231 molecules, cells were fixed with 2% paraformaldehyde in PBS for 15 min and then  
232 permeabilized with 0.04% Triton X100. Blocking was done with 5% BSA for 30 min at  
233 room temperature followed by staining with appropriate primary antibodies and  
234 fluorophore-conjugated secondary antibodies in 1% BSA for 1 hr. After each antibody  
235 incubation, cells were washed five times with PBS. Coverslips were mounted onto  
236 slides with ProLong Gold Antifade Reagent with DAPI. To study the colocalization of  
237 internalized FITC-VLP and ATP5B, the FITC-VLPs were bound to cells on ice for 1  
238 hr, washed with ice-cold PBS to remove unbound material and then shifted to 37°C  
239 to allow internalization for different times. Internalization was terminated by adding  
240 ice-cold PBS to cells. Surface bound VLPs were removed by first treating cells with  
241 trypsin-EDTA for 30 sec and then with surface stripping buffer (0.2% glacial acetic  
242 acid, 500 mM NaCl) for 1 min. The cells were fixed, permeabilized and stained with  
243 ATP5B primary antibody and Alexa Fluor 568 conjugated secondary antibody. All  
244 images were acquired on a Nikon A1R confocal laser scanning microscope (cLSM).  
245 Images were processed using Image J (<http://rsbweb.nih.gov/ij/download.html>) and  
246 colocalization coefficients were calculated using the NIS-Elements Microscope  
247 Imaging Software.

248 **Plasmid DNA isolation.** All plasmids were prepared using the RBC HiYield Plasmid  
249 DNA kit (Real Biotech Corporation, Taiwan) or cesium chloride density gradient  
250 centrifugation using standard protocols (Sambrook et al., 1989).

251 **Flow cytometry.** To evaluate the binding of HEV-LPs or cell surface levels of ATP5B  
252 by flow cytometry, cells were scraped, washed twice with FACS buffer (PBS + 0.1%  
253 FCS), blocked and incubated on ice for 1 hr. Cells were then incubated with either  
254 HEV-LP for 1 hr on ice or with anti-ATP5B polyclonal antibody in FACS buffer for 2-3

255 hr at 4°C, and washed three times with FACS buffer. The HEV-LP bound cells were  
256 stained with anti-HEV capsid followed by Alexa Fluor 488 conjugated secondary  
257 antibody; the anti-ATP5B stained cells were further stained with an appropriate  
258 secondary antibody. Cells were washed and subjected to flow cytometric analysis.  
259 Appropriate isotype control antibodies were used as primary antibodies for all  
260 stainings.

261 For antibody and HEV-LP binding and blocking experiments, cells were incubated  
262 with anti-ATP5B (or isotype control) antibodies for 2 hr on ice or with HEV-LPs for 1  
263 hr on ice. After incubation, the cells were fixed, washed with FACS buffer and  
264 incubated with HEV-LPs for 1 hr or with ATP5B antibodies for 2 hr, respectively.  
265 Cells were then washed and stained with anti-HEV capsid antibody and a fluorophore  
266 conjugated secondary antibody and subjected to flow cytometry.

267 All flow cytometric measurements were carried out on a CyAn ADP flow cytometer  
268 (Dako Cytomation). Data were analysed using the Summit Software (Dako  
269 Cytomation) and histograms were made using FlowJo (Tree Star, Inc.). Data were  
270 plotted as a percentage of fluorescent cells relative to mock (DMSO or other) treated  
271 cells.

272 **Western blotting.** Isolated membrane proteins were run on 10 or 12% denaturing  
273 polyacrylamide gels containing SDS, transferred to nitrocellulose membranes  
274 (Advanced Microdevices, India), blocked with 5% w/v BSA for 2 hr at room  
275 temperature and probed with various primary (overnight at 4°C) and HRP conjugated  
276 secondary antibodies (30 min at room temperature) diluted in TBS-T containing 1%  
277 BSA. Blots were visualized using Immuno-Cruz Western Blotting Luminol Reagent  
278 (Santa Cruz Biotechnologies Inc.) and exposed to X-ray films.

279 **HEV infection and estimation of viral titres.** The stool of a HEV-infected patient  
280 was used to make a 10% suspension in PBS, which was clarified by centrifugation at  
281 10,000 x g for 20 min at 4°C. This was then filtered successively through 0.45 µm  
282 and 0.22 µm filters and stored in single-use aliquots at -80°C. To estimate titres, RNA  
283 was isolated from 150 µl of this suspension using the QIAamp Viral RNA kit (Qiagen)  
284 and subjected to one step RT-Real time PCR (SuperScript III Platinum One-Step  
285 qRT-PCR Kit w/ROX, Life Technologies) using HEV specific primers and probes  
286 (Jothikumar et al., 2006). A standard curve was generated by this method using  
287 known concentrations of *in vitro* transcribed HEV RNA. Cells were pre-treated with  
288 anti-ATP5B (or isotype control) antibodies for 1 hr, followed by infection with 2 million  
289 genome equivalents of the stool suspension per million cells for 5 hr, followed by  
290 three PBS washes. The cells were then cultured in virus maintenance medium  
291 (DMEM containing 2% FCS and 2 mM MgCl<sub>2</sub>) for 40 hr. Total cellular RNA was  
292 isolated using TriZol (Invitrogen) and subjected to qRT-PCR (described above) to  
293 determine titres.

294 **Lipid raft isolation.** Lipid rafts were isolated according to published protocols (Legler  
295 et al., 2005). Briefly, FITC-VLP and CtxB-Biotin were bound on ice to Huh-7 cells for  
296 1 hr and lysed in chilled MNE buffer (25 mM MES, pH 6.5, 150 mM NaCl, 2 mM  
297 EDTA) containing 1% Triton X100 using a Dounce homogenizer. Alternatively, cells  
298 were washed and shifted to 37°C for 1 hr after binding of FITC-VLP and CTxB-biotin.  
299 The lysate was mixed with an equal volume of 90% sucrose in MNE buffer and  
300 placed in the bottom of an ultracentrifuge tube. This was overlaid with two volumes of  
301 35% sucrose followed by 5% sucrose. The gradient was centrifuged at 175,000 x g  
302 for 20 hr in a SW41Ti rotor (Beckman). One mL fractions were collected from the top  
303 and subjected to western blotting for FITC-VLP and EGFR, the latter as a marker of

304 non-raft fractions. To probe for raft-enriched fractions, these were blotted on  
305 nitrocellulose membrane, blocked in 5% BSA/PBS/0.1% Tween 20 and probed with  
306 Streptavidin-HRP at a dilution of 1:5000.

307

## 308 **RESULTS**

309 **Preparation of HEV-LPs and identification of binding partners.** The region  
310 corresponding to amino acids 368-606 of the HEV capsid protein (p239) was cloned  
311 and expressed in *E. coli* as described in Materials and Methods. Increasing amounts  
312 of the purified protein were run on a reducing SDS-polyacrylamide gel, which  
313 migrated between the 26 and 34 kDa markers (Fig. 1a). The purified protein also  
314 assembled into 20-40 nm virus like particles, as visualized by electron microscopy  
315 (Fig. 1b); these are called the hepatitis E virus-like particles (HEV-LPs). Dynamic  
316 light scattering (DLS) studies and gel permeation chromatography were also used to  
317 characterize the HEV-LPs as detailed elsewhere (Holla et al., 2015). The purified  
318 protein eluted in the void volume of a TSK3000 silica gel column (separation range  
319 10-500 kDa), indicating that higher order structures were being formed, and DLS  
320 measurements showed the average diameter of the particles to be 34 nm. We then  
321 checked the ability of these HEV-LPs to bind to cells of hepatic (Huh-7) and non-  
322 hepatic (HEK293) origin by flow cytometry, and found the binding to be higher on the  
323 former compared to the latter (Fig. 1c). These cell lines are considered permissive  
324 and non-permissive for HEV, respectively. Additionally, compared to HEK293 cells,  
325 the binding of HEV-LPs was higher on two other cell lines that are permissive for  
326 HEV infection (Takahashi et al., 2012) - PLC/PRF/5 (hepatoma) and A549 (lung  
327 carcinoma) (Fig. S1).

328 The C-terminal 6X Histidine tag present on the p239 monomer was used to  
329 immobilize the HEV-LPs to Ni-NTA beads. Membrane fractions from HEK293 and  
330 Huh-7 cells were passed over the immobilized HEV-LPs, the interacting proteins  
331 were eluted and resolved by SDS-PAGE. Two bands corresponding to 56.5 kDa and  
332 68.5 kDa were observed for both the cell lines (Fig. 1d). The bands were excised,  
333 subjected to in-gel tryptic digestion, and the proteins were identified by mass  
334 spectrometry to be ATP synthase subunit  $\beta$  (ATP5B) and Ribophorin I (RPN I) (Table  
335 S1).

336 **ATP5B is present on the surface of Huh-7 and HEK293 cells.** To ensure that  
337 ectopic (and not mitochondrial) ATP synthase was obtained from the pull-down  
338 assay, we isolated the total cell surface proteins from Huh-7 and HEK293 cells, and  
339 subjected these fractions to in-sol tryptic digestion and MALDI-TOF spectrometry.  
340 ATP5B was identified from Huh-7 cells, and not from HEK 293 cells (Table S2).  
341 Additionally, the alpha subunit (ATP5A) was also identified from Huh-7 cells,  
342 indicating that the two subunits may be forming a larger complex on the cell surface  
343 We also studied the localization and expression levels of ATP5B by flow cytometry  
344 and western blotting. Unpermeabilized Huh-7 and HEK293 cells were surface stained  
345 with ATP5B antibodies and subjected to flow cytometry. The ATP5B levels were  
346 higher on Huh-7 cells as compared to HEK293 cells (Fig. 1e;  $p < 0.01$ ), and this was  
347 consistent with HEV-LP binding to these cells (Fig. 1c). We also subjected  
348 membrane fractions from Huh-7 and HEK293 cells to western blotting for ATP5B. A  
349 band corresponding to ATP5B was observed in membrane fractions from Huh-7 and  
350 HEK293 cells, but the levels were significantly higher in the former as compared to  
351 the latter (Fig. 1f). Total cell lysates from Huh-7 and HEK293 cells showed high  
352 levels of ATP5B in both cell types. To ensure that membrane fractions were devoid of

353 cytoplasmic contaminants, they were probed for Actin and EGFR was used as a  
354 loading control. Plasma membrane fractionation protocols are subject to  
355 contamination from internal membranes. To ensure that ATP5B was not a  
356 contaminant from internal membranes, we carried out sucrose density gradient  
357 centrifugation and probed the collected fractions with antibodies against various  
358 subcellular markers: EGFR (plasma membrane), Calnexin (endoplasmic reticulum)  
359 and Cytochrome C (mitochondria) (Fig. S2). The ATP5B protein was found  
360 quantitatively in the EGFR marked plasma membrane fractions, with no visible  
361 contamination from internal membranes (ER, mitochondria) as seen from the lack of  
362 Calnexin and Cytochrome C in these fractions from both Huh-7 and HEK293 cells.

363 **Blocking of ATP5B reduces HEV-LP binding and virus infection.** We then  
364 checked whether antibody-mediated masking of cell surface exposed ATP5B  
365 affected the binding of HEV-LPs. For this, Huh-7 cells were pre-incubated with anti-  
366 ATP5B antibodies on ice, followed by the binding of HEV-LP, staining with anti-  
367 capsid antibodies and quantitation of binding with flow cytometry. The pre-incubation  
368 of cells with anti-ATP5B antibodies reduced the binding of HEV-LPs compared to  
369 cells treated with an isotype control antibody (Fig. 2a, 2b;  $p < 0.005$ ). Conversely,  
370 when Huh-7 cells were first incubated with HEV-LPs, fixed and subsequently stained  
371 with anti-ATP5B antibodies, the detectable surface levels of ATP5B were significantly  
372 reduced (Fig. 2c, d;  $p < 0.05$ ). To further validate these findings, we infected antibody  
373 pre-treated Huh-7 cells with HEV and estimated the virus in these cells two days  
374 post-infection by quantitative RT-PCR as described in Materials and Methods. The  
375 viral titers were found to be reduced by 1.5-2  $\log_{10}$  levels in cells pre-treated with  
376 ATP5B antibodies compared to the isotype control antibody (Fig. 2e,  $p < 0.01$ ).



377 The binding of HEV-LP was also checked on cells transfected with siRNA against  
378 ATP5B. Knockdown of total cellular levels of ATP5B were checked in siRNA  
379 transfected cells by western blotting. There was about 5-fold and 1.5-fold reduction in  
380 ATP5B levels in Huh-7 and HEK293 cells, respectively (Fig. 3a). This was also  
381 reflected in the levels of ectopic ATP5B on surface staining of siRNA transfected  
382 cells with anti-ATP5B antibodies. In Huh-7 cells there was a significant reduction in  
383 ectopic ATP5B levels (Fig. 3b;  $p < 0.01$ ), and even though HEK293 cells also showed  
384 reduced ectopic ATP5B levels, the difference was not significant (Fig. 3c). As  
385 expected, HEV-LP binding was significantly reduced in Huh-7 and HEK293 cells that  
386 received ATP5B siRNA compared to scrambled siRNA transfected cells (Fig. 3d, 3e).  
387 The extent of inhibition was greater for Huh-7 cells compared to HEK293 cells. As in  
388 the case of antibody-mediated block, we wanted to test siRNA transfected cells for  
389 HEV infection. However, repeated attempts at this were unsuccessful. Unlike HEV-  
390 LP binding, the HEV infection protocol required siRNA transfected cells to be tested  
391 for virus two days after infection. This extended incubation of cells knocked down for  
392 an essential protein such as ATP synthase, made these cells non-viable.

393 **Colocalization and intracellular trafficking of HEV-LP and ATP5B.** Viruses bind  
394 to surface receptors and are internalized along with the receptor in the form of a  
395 complex. We therefore asked whether cell surface bound HEV-LPs colocalized with  
396 ectopic ATP5B. For this, the HEV-LPs were labelled with FITC as described in  
397 Materials and Methods; these particles are called FITC-VLPs. Saturating  
398 concentrations of FITC-VLPs were bound to Huh-7 cells on ice for one hour (to  
399 prevent internalization of the FITC-VLP) and the cells fixed and counter-stained with  
400 anti-ATP5B antibody. The colocalization of surface bound FITC-VLPs was observed  
401 with cell surface ATP5B (Fig. 4a;  $n=22$ , Pearson's coefficient=0.597). Alternatively,

402 FITC-VLP bound cells were shifted to 37°C to allow internalization, and colocalization  
403 was checked at different times post-warming to determine if the internalized FITC-  
404 VLPs remained associated with ATP5B. For this, the cells were harvested at various  
405 times after FITC-VLP binding and internalization, surface stripped, fixed,  
406 permeabilized and stained with anti-ATP5B antibody. The internalized FITC-VLPs  
407 remained associated with ATP5B at 30 and 60 min, but the association was lost by 2  
408 hours post-internalization (Fig. 4b, 4c, 4d). Binding of virus or HEV-LP to receptor  
409 molecules is likely to trigger internalization of the virus-receptor complex into internal  
410 compartments such as endosomes, and the internalized FITC-VLPs would be  
411 expected to remain associated with ATP5B up to a certain time, before dissociating  
412 from it.

413 **Surface bound and internalized HEV-LPs are present in cholesterol-rich**  
414 **microdomains.** Ectopic ATP synthase has been shown to localize to cholesterol rich  
415 microdomains of the plasma membrane, also known as lipid rafts (Kim et al., 2004;  
416 Wang et al., 2006). These regions are enriched in molecules such as the GM1  
417 ganglioside receptor and some integrins. Cholera toxin subunit B (CTxB) binds GM1  
418 and is used as a marker for these membrane microdomains. To check if the HEV-LP  
419 was associated with lipid rafts during entry, we carried out binding of FITC-VLP and  
420 biotinylated CTxB to cells on ice for 1 hr, washed the cells and isolated lipid rafts  
421 using cold Triton extraction and sucrose density gradient centrifugation (Legler et al.,  
422 2005). Raft regions were detected by probing the fractions with Streptavidin-HRP.  
423 The surface bound FITC-VLP was found to partition to GM1-positive (raft enriched)  
424 as well as to EGFR-positive (non-raft enriched) fractions, although most of it was in  
425 the raft fractions (Fig. 5a). When FITC-VLP was allowed to internalize at 37°C for 1  
426 hr after binding on ice, it remained associated with the GM1-positive fractions,

427 although a significant portion was redistributed to the EGFR-positive non-raft regions  
428 (Fig. 5b). Since the VLP co-trafficks with ATP5B up to 1 hour post internalization  
429 (Figure 4), we conclude that some part of the internalized VLP remains associated  
430 with internalized ATP5B in the raft regions.

431 This is further corroborated by recent studies from our laboratory (Holla et al., 2015),  
432 which show that reducing membrane cholesterol levels with Nystatin/Progesterone or  
433 Filipin also inhibited the entry of HEV-LPs and HEV infection of Huh-7 cells.

434

## 435 **DISCUSSION**

436 Viruses bind to a diverse array of molecules on the surface of host cells to initiate  
437 infection. While some of these molecules are more generalized attachment factors  
438 that concentrate the virus on the cell surface, productive entry usually requires  
439 binding to one or more specific receptors. We have previously shown that HSPGs,  
440 specifically Syndecans, are critical attachment factors on liver cells that bind HEV  
441 (Kalia et al., 2009). However, the cellular receptor(s) for HEV, a virus that is endemic  
442 to large parts of the world and causes significant morbidity and mortality (Khuroo,  
443 2011), have so far not been identified. The reasons for this and other lacunae in the  
444 understanding of HEV biology is its inability to grow efficiently in culture or to suitably  
445 infect small animals (Tanaka et al., 2007). We have circumvented this by utilizing a  
446 recombinant hepatitis E virus-like particle (HEV-LP) to identify potential cellular  
447 receptor(s), followed by confirmation with infectious virions.

448 Two cellular proteins, ATP synthase subunit  $\beta$  (ATP5B) and Ribophorin I (RPN1)  
449 were identified using a HEV-LP mediated pull down of plasma membrane proteins.

450 The results were counter-intuitive since both of these molecules are not classically  
451 present on the cell surface. The ATP5B is part of the mitochondrial  $F_1$ - $F_0$  ATPase but  
452 is also found on the plasma membrane (Chi & Pizzo, 2006) ; RPN1 is part of the  
453 oligosachcharyltransferase complex in the rough endoplasmic reticulum (RER) and is  
454 involved in the N-glycosylation of proteins (Helenius & Aebi, 2004). Although RPN1  
455 has not been reported to be present on the cell surface, our flow cytometric analyses  
456 and western blotting of plasma membrane fractions do indicate this (data not shown).

457 The ATP5B contains the catalytic core of the large multi-subunit ATP synthase  
458 complex, which drives ATP synthesis in the mitochondria (Boyer, 1997).  
459 Mitochondrial ATP synthase is translocated to cholesterol-rich 'lipid raft' regions of  
460 the plasma membrane, but the mechanism of this translocation is not understood  
461 (Kim et al., 2004). Further, adding cholesterol to HUVEC cells increased  
462 translocation of the ATP synthase to the plasma membrane (Wang et al., 2006).  
463 Surface localized or 'ectopic' ATP synthase is involved in the maintenance of  
464 intracellular pH, cholesterol homeostasis and cellular response to angiogenic factors  
465 (Chi & Pizzo, 2006), influenza virus maturation and budding (Gorai et al., 2012), and  
466 the transfer of HIV-1 particles from antigen presenting cells (APCs) to CD4+ T cells  
467 at the virological synapse (Yavlovich et al., 2012). Recently, ATP5B has also been  
468 shown to be involved in the entry of the Chikungunya virus (CHIKV) into insect cells  
469 (Fongsaran et al., 2014).

470 Since ectopic ATP synthase plays important roles in the entry and morphogenesis of  
471 other viruses, we investigated its role as an entry factor for HEV. Through a series of  
472 experiments that involved characterization of plasma membrane fractions, surface  
473 staining for ATP5B by flow cytometry, and mass spectrometric profiling of the surface

474 proteome from different cell lines, we have shown that ATP5B is indeed present on  
475 the plasma membrane and its levels are higher in cells that are permissive for HEV  
476 infection compared to those that are not. However, it was interesting to find ATP5B in  
477 the p239-mediated pull down from both permissive (Huh-7) and non-permissive  
478 (HEK293) cells, although its levels were consistently higher in the former. This could  
479 be due to pull down using purified membrane fractions, where the concentrations of  
480 ATP5B were much higher as compared to whole cells. Additionally, the stoichiometry  
481 of binding of these membrane proteins to an immobilized VLP *in vitro* would be  
482 different from that on the cell surface. The permissive (Huh-7, PLC/PRF/5, A549) and  
483 non-permissive (HEK293) nature of cell lines might also be due to factors other than  
484 the presence or absence of a cell surface molecule that the virus uses for entry. For  
485 example, coxsackieviruses group B (CVB), bind CHO cells due to the presence of  
486 CAR receptors, although replication does not take place in these cells (Kramer et al.,  
487 1997) Similarly, most tissue macrophages are considered permissive for HIV-1  
488 infection, since these are derived from monocytes. However, intestinal macrophages  
489 do not support HIV-1 infection. Exposure of monocytes to intestinal extracellular  
490 matrix (stroma)-conditioned media (S-CM) and HIV-1 simultaneously, revealed that  
491 the CD4+ CCR5+ subset of differentiated intestinal macrophages did not support  
492 HIV-1 infection, indicating that factors other than receptor binding may be involved in  
493 making cells permissive or otherwise (Shen et al., 2011).

494 Huh-7 cells pre-incubated with antibodies against ATP5B showed reduced HEV-LP  
495 binding; conversely, surface staining for ATP5B was also significantly reduced when  
496 HEV-LP was pre-bound to cells. The knockdown of ATP5B with siRNA also led to  
497 reduced HEV-LP binding to Huh-7 cells. During internalization of surface-bound  
498 HEV-LPs, these were found to colocalize with ATP5B for up to one hour post-

499 internalization, following which this association was lost. The internalized HEV-LPs  
500 also colocalized with CTxB, which binds the GM1 receptor and is a marker for  
501 cholesterol-enriched membrane microdomains or 'lipid rafts' (Torgersen et al., 2001).  
502 This was also the case when these membrane microdomains were prepared using  
503 cold Triton extraction and sucrose gradient centrifugation. This could be due to  
504 binding of the HEV-LPs to the ectopic ATP synthase complex, which resides in these  
505 membrane fractions (Kim et al., 2004). To validate our findings, surface exposed  
506 ATP5B was blocked with specific antibodies and the cells were infected with virions  
507 from the stool of an infected hepatitis E patient. Intracellular virus replication was  
508 compromised in Huh-7 cells pre-treated with anti-ATP5B antibodies. This reduced  
509 replication is likely to be due compromised HEV binding and internalization, and  
510 validates our findings that ATP5B is involved in the binding and entry of HEV into  
511 liver cells.

512

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517

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699

700 **FIGURE LEGENDS**

701 **Figure 1: (A)** The HEV-LP was expressed in *E.coli* BL21 (DE3) and purified from  
702 inclusion bodies. Increasing concentrations (7.5 to 30  $\mu$ g) of purified recombinant  
703 protein were run on a SDS-12% polyacrylamide gel and visualized by Coomassie  
704 Blue staining. **(B)** Electron microscopic image of the HEV-LP preparation was  
705 obtained by loading purified protein onto Formvar-coated copper grids and visualizing  
706 at 97,0000x magnification. **(C)** Different amounts of HEV-LP (100 ng to 10  $\mu$ g) were  
707 bound to Huh-7 or HEK293 cells, stained with anti-HEV capsid antibody, and  
708 subjected to flow cytometry. Data represents mean fluorescence intensity from three  
709 independent experiments. **(D)** Membrane proteins from Huh-7 and HEK293 cells  
710 were used in a pull-down experiment to identify HEV entry factors. Eluates were  
711 separated on an SDS-polyacrylamide gel and stained with Coomassie Blue. Two  
712 bands of the indicated molecular sizes were observed. Positions of protein marker  
713 (M) bands are shown on the left. **(E)** Surface levels of ATP5B were measured on  
714 Huh-7 and HEK293 cells by staining cells with anti-ATP5B (or isotype control)  
715 antibodies followed by flow cytometry; data is representative of three independent  
716 experiments  $\pm$  SD (\*\* $p < 0.01$ ). **(F)** Membrane protein fractions and total cell lysate  
717 from Huh-7 and HEK293 cells were separated on a SDS-12% polyacrylamide gel,  
718 transferred to nitrocellulose membrane and probed for ATP5B using specific  
719 antibodies. Membrane fractions were probed for Actin to check for cytoplasmic  
720 contamination and EGFR was used as a loading control. Protein markers are  
721 indicated.

722 **Figure 2: (A)** Huh-7 cells were incubated with anti-ATP5B (or isotype control)  
723 antibodies, following which HEV-LP (50  $\mu$ g) was bound to cells. Cells were stained



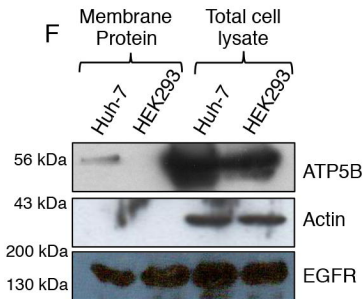
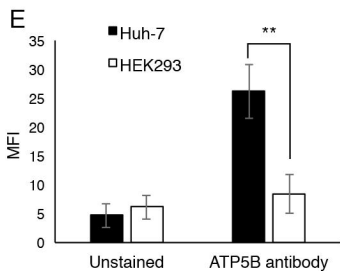
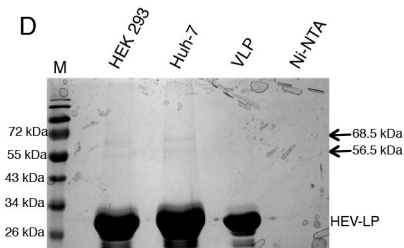
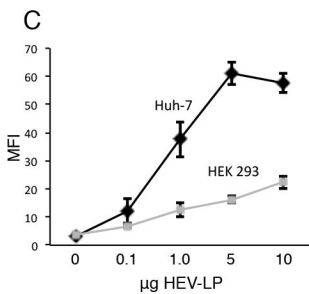
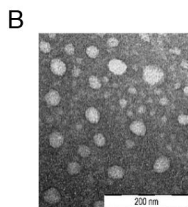
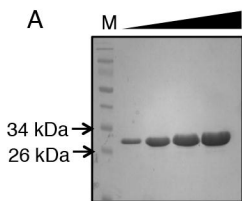
724 with anti-HEV capsid antibody and subjected to flow cytometric analysis. Histograms  
725 were plotted using FlowJo software. **(B)** The mean fluorescence intensity from the  
726 previous experiment was plotted. Data represents average of three independent  
727 experiments  $\pm$  SD \*\*\* $p < 0.005$ . **(C)** HEV-LP (50  $\mu$ g) was bound to cells first, followed  
728 by staining for ATP5B. Flow cytometry was carried out and histograms were plotted  
729 using the FlowJo software. **(D)** Mean fluorescence intensity was plotted and data  
730 represents an average of three independent experiments  $\pm$  SD \* $p < 0.05$ . **(E)** HEV  
731 infection was carried out as described in Materials and Methods. Cells were  
732 incubated with anti-ATP5B (or isotype control) antibodies, HEV infection was carried  
733 out and 40 hr later HEV genome equivalents were estimated by qRT-PCR using a  
734 standard curve. Data are shown for three experiments  $\pm$  SD (\*\* $p < 0.01$ ).

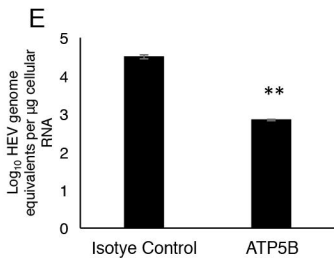
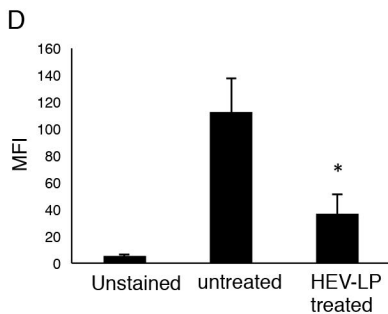
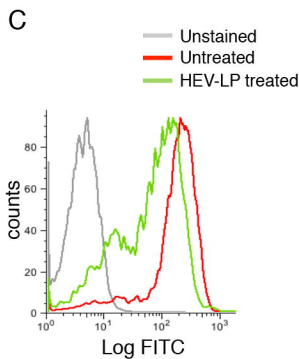
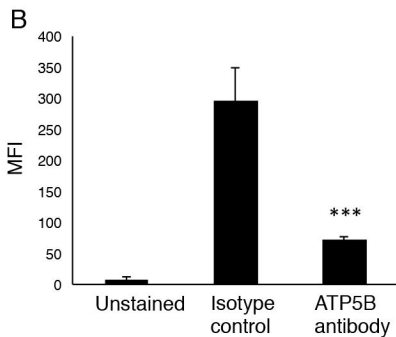
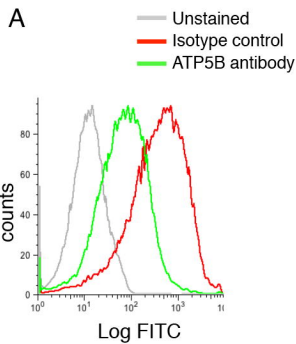
735 **Figure 3: (A)** Huh-7 or HEK293 cells were transfected with ATP5B-specific or  
736 scrambled control siRNAs as described in Materials and Methods. The extent of  
737 knockdown of total ATP5B in both cell lines was assayed by western blotting. Actin  
738 was used as a loading control. To check the knockdown of surface expressed  
739 ATP5B, Huh-7 cells **(B)** or HEK293 cells **(C)** were transfected with ATP5B-specific or  
740 scrambled control siRNA, fixed and stained with anti-ATP5B antibodies.  
741 Representative histograms plotted using FlowJo are shown on the left. Mean  
742 fluorescence intensity is shown on the right for both cell lines; data represents three  
743 independent experiments  $\pm$  SD (\*\* $p < 0.01$ ). HEV-LP binding was checked in Huh-7  
744 **(D)** or HEK293 **(E)** cells transfected with ATP5B-specific or scrambled control  
745 siRNAs. Representative histograms plotted using FlowJo are shown on the left and  
746 mean fluorescence intensities of three independent experiments  $\pm$  SD are shown on  
747 the right for both cell lines (\* $p < 0.05$ ; \*\*\* $p < 0.001$ ).

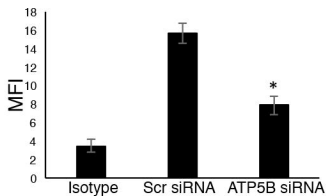
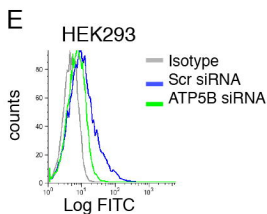
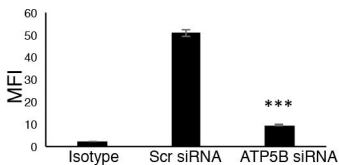
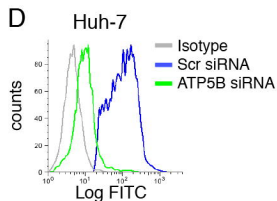
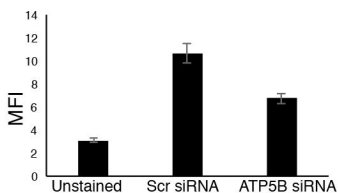
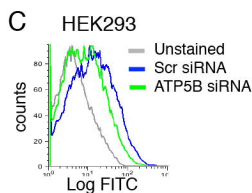
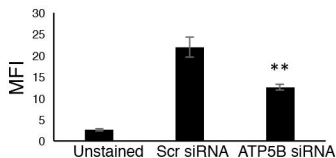
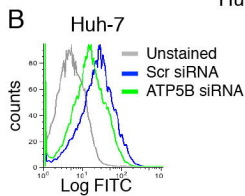
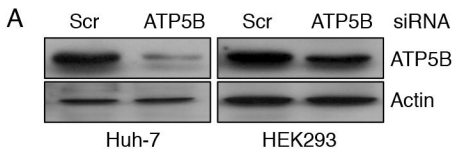
748 **Figure 4:** Colocalization and trafficking of HEV-LPs and ATP5B. **(A)** FITC-VLP  
749 (green) was bound to Huh-7 cells on ice, the cells were fixed and counter-stained  
750 with anti-ATP5B antibodies (red) and visualized by confocal microscopy. **(B)** FITC-  
751 VLP (green) was bound to cells on ice and was subsequently allowed to internalize at  
752 37°C. At the indicated times post-internalization, cells were fixed, permeabilized and  
753 stained for ATP5B (red), and visualized by confocal microscopy. The boxed regions  
754 have been enlarged on the right. Colocalization was quantified using the NIS  
755 Elements software. Scale bars are shown.

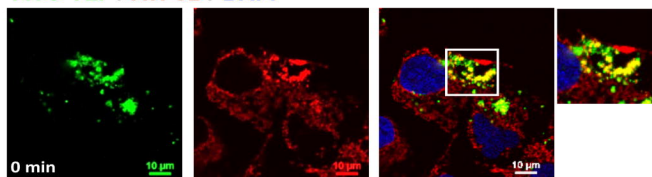
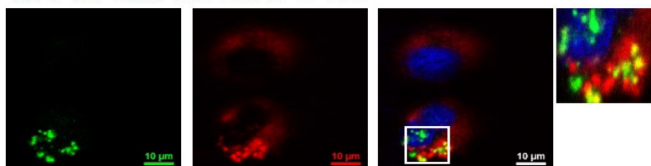
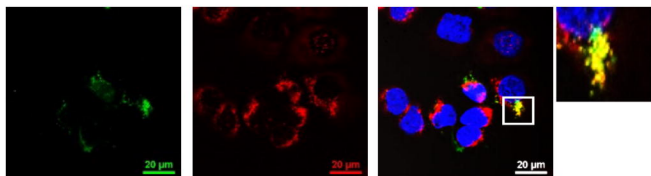
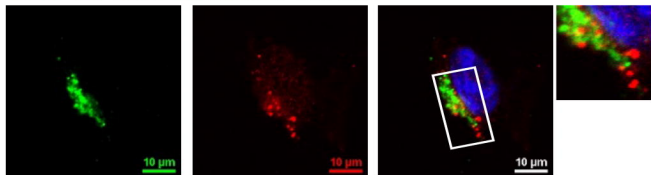
756 **Figure 5:** HEV-LPs bind lipid raft regions of the cell membrane. **(A)** FITC-VLP and  
757 biotinylated Cholera toxin subunit B (CTxB-Biotin) were allowed to bind to Huh-7 cells  
758 on ice for 1 hr, and cold Triton extraction was carried out followed by sucrose density  
759 gradient centrifugation as described in Materials and Methods. One mL fractions  
760 were collected from the top of the ultracentrifuge tube; 5 µL of each was blotted onto  
761 nitrocellulose membranes, blocked with BSA and probed with HRP-conjugated  
762 Streptavidin (bottom panel). Further, 50 µL from each fraction was run on a reducing  
763 SDS-polyacrylamide gel, transferred to nitrocellulose membrane and probed with  
764 antibodies to EGFR (a marker for non-raft membrane regions) or HEV-LP (ORF2  
765 protein) as indicated. **(B)** FITC-VLP was allowed to internalize at 37°C after binding  
766 on ice for 1 hr, and lipid rafts were isolated and probed as described above.

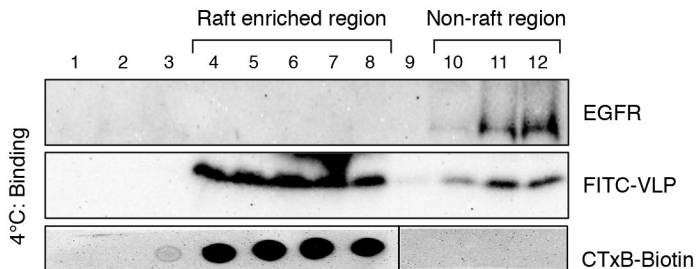
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**A****FITC-VLP / ATP5B / DAPI****B****FITC-VLP / ATP5B / DAPI / 30 min****FITC-VLP / ATP5B / DAPI / 60 min****FITC-VLP / ATP5B / DAPI / 120 min**

**A****B**