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EGF receptor modulates HEV entry in human hepatocytes

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Epidermal Growth Factor Receptor Modulates Hepatitis E Virus Entry in Human Hepatocytes

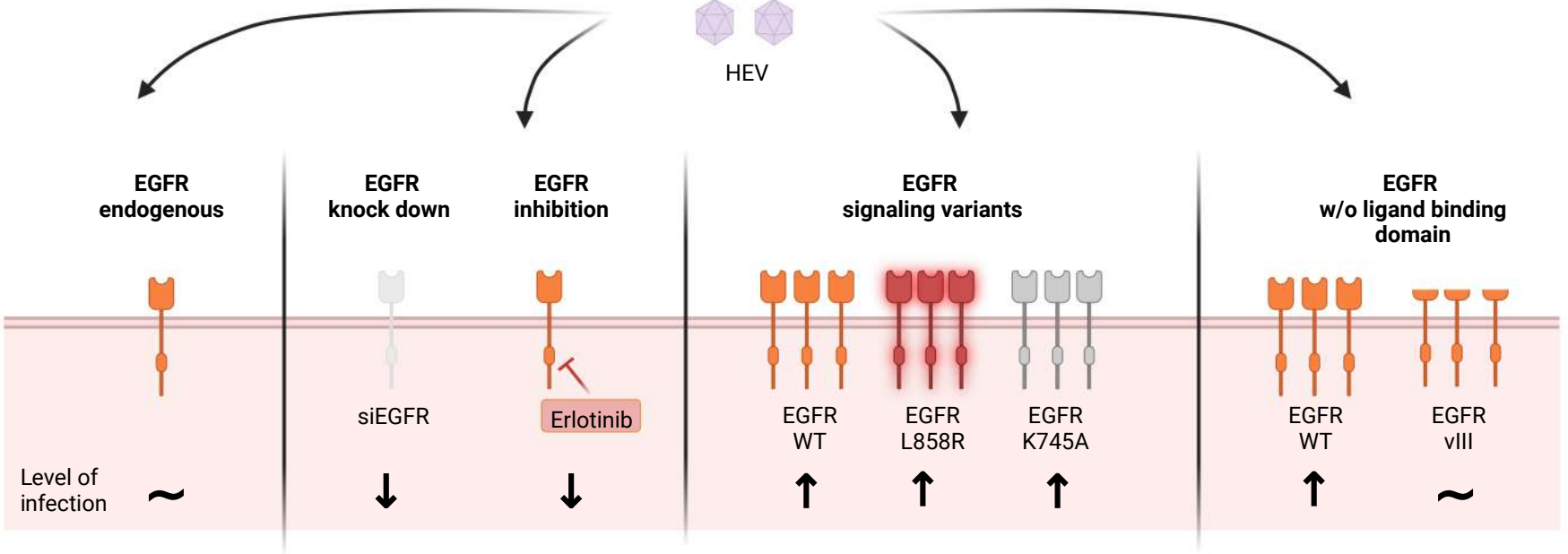
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	Infection Research (DZIF), External Partner Site
Keywords:	HEV, EGFR, viral entry, life cycle, host factor



Hepatitis E virus (HEV) entry



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3 **1 Epidermal Growth Factor Receptor Modulates Hepatitis E Virus Entry in Human**
4 **2 Hepatocytes –HEP-22-1113**
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10 4 Jil Alexandra Schrader¹, Thomas Leon Burkard¹, Yannick Brüggemann¹, André Gömer¹, Toni Luise
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29 52 host factor

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3 **63 List of Abbreviations**
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5 64 Cetu Cetuximab
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7 65 EGF Epidermal Growth Factor
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9 66 EGFR Epidermal Growth Factor Receptor
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11 67 Erlo Erlotinib
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13 68 FFU Focus Forming Units
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15 69 fl Full length
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17 70 HAV Hepatitis A virus
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19 71 HBV Hepatitis B virus
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21 72 HCV Hepatitis C virus
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23 73 HEV Hepatitis E virus
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25 74 HEVcc Cell culture derived HEV
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27 75 Gluc *Gaussia* luciferase
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29 76 PHH Primary Human Hepatocytes
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31 77 Rbv Ribavirin
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33 78 RLU Relative light units
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35 79 SGR Subgenomic Replicon System
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37 80 UTC Untreated control
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39 81 WT Wildtype
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3 84 **Abstract:**
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5 85 **Background and Aims:** Being the most common cause for acute viral hepatitis with more than
6
7 86 20 million cases per year and 70 000 deaths annually, hepatitis E virus (HEV) presents a long neglected
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9 87 and under-investigated health burden. Although the entry process of viral particles is an attractive target
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11 88 for pharmacological intervention, druggable host factors to restrict HEV entry have not been identified
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13
14 89 so far.

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16 90 **Approach and Results:** Here we identify the epidermal growth factor receptor (EGFR) as a novel host
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18 91 factor for HEV and reveal the significance of EGFR for the HEV entry process. By utilizing RNAi,
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20 92 chemical modulation with FDA-approved drugs and ectopic expression of EGFR, we revealed that
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22 93 EGFR is critical for HEV infection, without affecting HEV RNA replication or assembly of progeny
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24 94 virus. We further unveiled that EGFR itself and its ligand binding domain rather than its signaling
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26 95 function is responsible for the proviral effect. Modulation of EGF expression in HepaRG cells as well
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28 96 as primary human hepatocytes affected HEV infection.

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32 97 **Conclusions:** Taken together, our study provides novel insights into the life cycle of HEV and identified
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34 98 EGFR as a possible target for future anti-viral strategies against HEV.
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100 Introduction:

101 Hepatitis E virus (HEV) is the main cause of acute viral hepatitis creating endemic, waterborne
102 outbreaks in developing countries and increasing zoonotic danger in the developed world with estimated
103 20 million infections leading to 3.3 million symptomatic cases and 44 000–70 000 deaths per year ¹.
104 Clinical manifestations of HEV infections range from mild symptoms of acute hepatitis to fulminant
105 hepatitis and chronic infections in immunocompromised patients along with a fatality rate of up to 30%
106 in pregnant women ^{2,3}. In Europe, known cases of HEV infections have increased 10-fold from 2005
107 until 2015, with some countries even reporting exceeding cases of HEV than hepatitis A virus (HAV),
108 indicating that further studies into HEV's pathophysiology is urgently needed ⁴. HEV is a quasi-
109 enveloped, positive sense, single-stranded (ss+) RNA virus of the genus *Paslahepevirus* within the
110 family of *Hepeviridae* ⁵⁻⁷. HEV's icosahedral, 27–34 nm virion encapsulates a 7.2 kb RNA, which
111 consists of three open reading frames (ORFs 1–3) encoding the viral replicase, viral capsid and a protein
112 for which multiple functions have been described, respectively ^{6,8,9}. Since a robust cell culture system
113 has been developed only recently¹⁰, little is known about molecular determinants involved in HEV's
114 entry and life cycle progression ¹¹⁻¹³. First evidence suggest that as its first attachment, the HEV ORF2
115 capsid protein interacts with heparan sulfate proteoglycans on the cell surface ¹⁴. After binding to a yet
116 unknown receptor, the virion is endocytosed in a clathrin-dependent manner ^{14,15}. Integrin- α 3 has
117 recently been discovered as a putative host factor for the entry of non-enveloped HEV particles ¹⁶.
118 However, the role of Integrin- α 3 for the HEV entry is still poorly understood and additional host factors
119 may be required for HEV entry into the host cell.

120 The epidermal growth factor receptor (EGFR) has been demonstrated to be critical for the entry of a
121 number of hepatotropic viruses, including hepatitis B virus (HBV) and hepatitis C virus (HCV) ^{17,18}.
122 EGFR is a receptor tyrosine kinase of the ErbB family, which controls cell proliferation, migration, and
123 differentiation ¹⁹. Upon ligand binding to the receptor's extracellular ligand-binding domain, EGFR
124 forms asymmetric homo- and heterodimers with other ErbB family members in which one kinase
125 domain brings the other kinase domain into an active state catalyzing autophosphorylation of multiple
126 tyrosine residues in the C-terminal domain ²⁰⁻²². Consequently, binding sites for adaptor proteins are

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3 127 created initiating the downstream signaling cascade that contains a network of around 200 proteins
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5 128 relaying on the extracellular signal inside the cell²³. After ligand binding, EGFR is internalized by
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7 129 either clathrin-dependent or -independent endocytosis to endosomes and either routed for degradation
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9 130 or recycling^{24–26}. Several mechanisms by which EGFR can facilitate numerous different viral infections
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11 131 have been described so far, including enabling viral entry into host cells^{27,26}. In the context of HCV
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13 132 infections, EGFR acts as co-factor regulating interactions between the entry receptors CD81 and
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15 133 claudin-1 and the subsequent fusion with host cell membranes in a clathrin-dependent manner²⁸.
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18 134 In this study, we examined the role of EGFR and its signaling during HEV infection. Through siRNA-
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20 135 mediated knockdown of EGFR, chemical inhibition and modulation of EGFR by FDA-approved drugs,
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22 136 as well as ectopic expression of the receptor, we identified EGFR as a novel HEV host factor required
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24 137 during viral entry. Additionally, the ability of EGFR modulators to effectively suppress HEV infection
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26 138 in the authentic cell culture models such as HepaRG cells and notably primary human hepatocytes
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28 139 suggested that EGFR also plays a key role during HEV infection. Taken together, our study provides
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30 140 novel insights into the life cycle of HEV and identified a possible target for future anti-viral strategies
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32 141 against HEV.
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143 **Materials and Methods**

144 *Cell culture*

145 The human hepatoma cell line HepG2 (ATCC-Nr.: HB-8065) and 293T cells (ATCC-Nr.: CRL-3216)
146 were cultured in Dulbecco's Modified Eagle's Medium (DMEM-high-glucose, Gibco, Cat.Nr. 11965)
147 supplemented with 10% (v/v) fetal calf serum (FCS, Capricorn, Lot.Nr. CPC21-4114), 1% (v/v) non-
148 essential amino acids (NEAAs, Gibco, Cat.Nr. 11140050), 100 IU/mL penicillin, 100 µg/mL
149 streptomycin (Gibco, Cat.Nr. 15140) and 2 mM L-glutamine (Gibco, Cat.Nr. 25030). For virus titration,
150 a HepG2-subclone (HepG2/C3A) was used due to its greater infection efficiencies cultured in Eagle's
151 minimum essential medium (MEM, Gibco, Cat.Nr.11095) supplemented with 10% (v/v) ultra-low IgG-
152 FCS (Gibco, Cat.Nr. 16250-078, Lot 1939770), 100 µg/mL gentamicin (Gibco, Cat.Nr. 15710), 2 mM
153 L-glutamine, 1 mM sodium pyruvate (Gibco, Cat.Nr. 11360) and 1% (v/v) NEAAs. HepG2 and
154 HepG2/C3A cells were grown on rat collagen-coated (SERVA Electrophoresis, Cat.Nr. 47256.01) cell
155 culture dishes. As previously described²⁹, undifferentiated HepaRG cells were cultured in HepaRG
156 growth medium consisting of William's Medium E (Gibco, Cat.Nr. 22551), supplemented with 10%
157 (v/v) FCS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL gentamicin, 2 mM L-alanyl-L-
158 glutamine dipeptide (GlutaMax, Gibco, Cat.Nr. 35050), 5 µg/mL insulin (Sigma-Aldrich, Cat.Nr.
159 I9278) and 50 mM hydrocortisone hemisuccinate (Sigma-Aldrich, Cat.Nr. 1319002). For
160 differentiation, 5×10^4 HepaRG cells were seeded on 24-well plates and incubated for 14 days, followed
161 by 14 days incubation in HepaRG growth medium supplemented with 1.8% (v/v) dimethyl sulfoxide
162 (DMSO Hybri-Max, Sigma-Aldrich, Cat.Nr. D2650). Medium was changed twice a week. Primary
163 human hepatocytes (PHH) were prepared from non-tumorous tissue obtained from freshly resected
164 livers as previously described^{30,31}. Written informed consent was obtained from all patients, and the
165 study was approved by the institutional review board (Ethics Committee) of the medical faculty at the
166 University Duisburg-Essen. Human biological samples were provided by the Westdeutsche Biobank
167 Essen (WBE, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; approval 18-
168 WBE-048). PHHs were seeded into collagen I-coated culture plates and cultured in William's
169 Medium E supplemented with 5% (v/v) FCS, 1% (v/v) NEAAs, 100 IU/mL penicillin, 100 µg/mL

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3 170 streptomycin, 2 mM GlutaMAX, 2% (v/v) DMSO, 10 mM HEPES (Gibco, Cat.Nr. 15630), 5.4 μ M
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5 171 hydrocortisone hemisuccinate, 5.5 ng/mL EGF (human, Med Chem Express, HY-P7109), and 5 μ g/mL
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7 172 insulin. All cells were kept at 37 °C in a 5% (v/v) CO₂ incubator.
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12 174 All materials and methods describing virus production and all assays utilized in this study are specified
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14 175 in the supplementary information.
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Results*EGFR is abundantly expressed in human hepatocytes*

Numerous hepatotropic viruses, such as the hepatitis B virus (HBV) and the hepatitis C virus (HCV), have been shown to exploit the epidermal growth factor receptor (EGFR) during the virus entry process^{17,18}. Since very little is known about host factors for hepatitis E virus (HEV), we aimed to investigate the role of EGFR and its signaling during HEV infections.

To determine the expression of EGFR in hepatocytes, we analyzed single-cell RNA-sequencing data of the human liver cell atlas from nine healthy human donors³². T-distributed-stochastic-neighbor-embedding (t-SNE) plots highlighted the mRNA expression of the hepatocyte marker Albumin (Fig. 1A). Similar to reported HEV host factors TSG101 and Rab5, EGFR was highly abundant in clusters of hepatocytes and cholangiocytes (Fig. 1B).

Endogenous EGFR is critical for HEV infection

Given that hepatocytes are the main target of HEV during infection, we aimed to address the role of endogenous EGFR upon HEV infection. Therefore, we performed siRNA-mediated knockdown followed by HEV infection in HepG2 cells. The reduction of EGFR protein expression was confirmed via western blot and immunofluorescence staining (Fig. 2A). Next, we infected EGFR knockdown and control HepG2 cells with HEVcc (p6) followed by immunofluorescence and FFU determination. We observed that silencing of EGFR reduced the number of HEV infection events by approximately 50%, demonstrating the significance of endogenous EGFR during HEV infection (Fig. 2B). In order to confirm this data in a more authentic cell culture system, we used induced pluripotent stem cell (iPSC)-derived hepatocyte like cells (HLCs) and transduced these with short hairpin RNA targeting EGFR (shEGFR), followed by infection with HEVcc (p6). The silencing of EGFR via shEGFR in HLCs was confirmed via western blot (supplementary Fig. S1A). The ratio of ORF2 protein positive (ORF2+) and transduced cells was lowered in shEGFR transduced cells compared to shCtrl cells (supplementary Fig. S1B–C) confirming the crucial role of endogenous EGFR during HEV infection.

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3 202 EGFR can be inhibited and modulated by multiple FDA-approved drugs, including Erlotinib (Erlo), an
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5 203 EGFR-specific tyrosine kinase inhibitor, as well as Cetuximab (Cetu), an antibody that competitively
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7 204 binds to the extracellular receptor domain and hinders receptor dimerization, thus impeding signal
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9 205 transduction. We aimed to explore the potential of these molecules as antivirals to combat HEV
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11 206 infection. First, we confirmed the ability of Erlotinib and Cetuximab to inhibit EGFR phosphorylation
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13 207 at the concentrations used in the following assays by immunofluorescence analysis of pEGFR(-1068)
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15 208 in serum starved, EGFR modulator treated HepG2 cells (supplementary Fig. S2). EGF, the cognate
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17 209 ligand of EGFR served as positive control to induce EGFR phosphorylation (supplementary Fig. S2).
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19 210 Subsequently, we infected hepatoblastoma cells with non-enveloped HEVcc of different strains (p6 and
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21 211 83-2) as well as enveloped HEVcc (p6) in the presence or absence of different EGFR-specific
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23 212 modulators for the whole time of infection (5 days) (Fig. 2C–D and supplementary Fig. S3. Treatment
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25 213 with the FDA-approved, EGFR-specific tyrosine kinase inhibitor Erlotinib resulted in a reduction of
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27 214 HEV p6,83-2 and enveloped p6 infection events by approximately 76%, 99% and 97% respectively.
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29 215 Also, EGF, as well as Cetuximab reduced HEV infection by 43% and 35% (p6), 59% and 69% (83-2)
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31 216 and 70% and 81% (enveloped p6), respectively. Of note, cell viability determined via MTT-assay was
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33 217 not affected by the applied concentration of the different EGFR modulators. In addition, Erlotinib was
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35 218 capable of inhibiting HEVcc p6 infections in a dose-dependent manner (supplementary Fig. S4). These
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37 219 data suggest that perturbation of endogenous EGFR can prevent HEV infection.
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221 *Endogenous EGFR is required during HEV entry*

222 To dissect which step of the HEV life cycle is affected upon inhibition of endogenous EGFR, we
223 performed different virological assays evaluating HEV attachment, entry, post-attachment, replication
224 and assembly of progeny viruses in the presence or absence of EGFR modulators.

225 To evaluate whether endogenous EGFR is critical for the entry process of HEV, HepG2 cells were
226 pretreated with the different modulators followed by infection with HEVcc (p6). The applied drugs and
227 virus inoculum were replaced with fresh medium after 8 h of incubation. Ribavirin (Rbv) was reapplied

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3 228 to serve as positive control for efficient inhibition of HEV replication. By subsequent FFU counting,
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5 229 we identified that Erlotinib treatment significantly reduced HEV infection when applied during HEV
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7 230 inoculation (Fig. 3A), implying that EGFR is critical for the entry process of HEV.
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10 231 In order to dissect whether the restriction of HEV entry by EGFR modulators was based on restriction
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12 232 of the HEV attachment to the target cells, we incubated modulator-treated HepG2/C3A cells with HEV
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14 233 on ice for 2 h, allowing virus attachment but not cell entry. Here, anti-HEV serum neutralized HEV
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16 234 particles, thus inhibiting the attachment of HEV. Unbound HEV was removed by repeated washing
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18 235 with PBS and cells were either directly lysed for RT-qPCR analysis or incubated for 3 days at 37 °C
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20 236 followed by FFU counting. Thereby, we observed that EGFR modulator treatment did not significantly
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22 237 alter HEV RNA copy numbers (supplementary Fig. S5A) nor the number of FFU per well (Fig. 3B),
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24 238 suggesting that modulation of endogenous EGFR does not influence the attachment of HEV particles.
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28 239 To address the role of EGFR on post-binding steps of HEV, we conducted a post-attachment assay by
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30 240 inoculating pre-cooled HepG2/C3A cells with HEV on ice for 2 h. Inoculum was removed and cells
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32 241 were treated with EGFR modulators for either 8 h post infection (p.i.) or 3 d p.i. at 37 °C. HEV infection
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34 242 was quantified at 3 d p.i., showing a significant reduction in HEV FFU per well in Erlotinib treated cells
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36 243 during HEV post-attachment for both 8 h as well as 3 d, while Ribavirin treatment reduced HEV
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38 244 infections only when treated for longer than 8 h (Fig. 3C). These data imply that endogenous EGFR
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40 245 modulation affects post-binding steps of HEV.
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43 246 To circumvent the HEV entry process and address possible effects of EGFR modulators on intracellular
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45 247 life cycle steps, we transfected *in vitro* transcribed (IVT) HEV-Gluc RNA. Hereby, we utilized the HEV
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47 248 RNA subgenomic replicon (SGR) system, carrying a luciferase reporter, to monitor HEV RNA
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49 249 replication. We detected that EGFR modulator treatment did not affect HEV RNA replication (Fig. 3D).
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51 250 To investigate potential effects of EGFR inhibitors on HEV progeny virus production, IVT full length
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53 251 HEV RNA was electroporated into hepatoblastoma cells. By quantification of progeny virus, we
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55 252 detected similar viral titers in the presence or absence of EGFR modulators (Fig. 3E), implying that
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57 253 EGFR modulators do not affect HEV RNA replication and virus assembly.
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3 254 Overall, our data show that endogenous EGFR affects the HEV entry process and has no effect on viral
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5 255 attachment, replication and assembly of progeny virus.
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8 256 *Ectopic EGFR expression facilitates HEV infection*
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11 257 To further evaluate the role of EGFR during HEV entry, we generated HepG2 cells ectopically
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13 258 expressing EGFR. After confirming the ectopic expression of EGFR via western blot and
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15 259 immunofluorescence staining (Fig. 4A), we challenged the cells with HEVcc (p6). Thereby, we
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17 260 observed an increase of HEV infection in the presence of EGFR compared to HepG2 empty vector cells
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19 261 (Fig. 4B). By utilizing the HEV RNA SGR and by producing HEVcc in these cells, we further
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21 262 confirmed that ectopically expressed EGFR rather facilitates initiation of HEV infection without
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23 263 affecting HEV RNA replication and progeny virus production (Fig. 4C middle and right panel).
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26 264 Given that modulation of endogenous EGFR restricted HEV entry, we next asked whether the proviral
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28 265 effect of ectopically expressed EGFR is sensitive to EGFR modulator treatment. Importantly, we
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30 266 identified that the proviral effect of EGFR overexpression could be reversed by applying the EGFR-
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32 267 specific modulators during HEV inoculation (Fig. 4C left panel and D). Similar to the inhibition of
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34 268 endogenous EGFR, we observed that especially Erlotinib restricted HEV infection in these assays.
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36 269 Furthermore, we performed an attachment assay, similar to Fig. 3B, with cells ectopically expressing
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38 270 EGFR and control cells to quantify HEV RNA copy numbers after viral inoculation at 4 °C for 2 h. We
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40 271 detected similar HEV RNA copy numbers in EGFR expressing cells and control cells indicating that
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42 272 EGFR does not affect HEV attachment (supplementary Fig. S5B). Taken together, our obtained data
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44 273 suggest that access to EGFR is a limiting parameter for initiation of HEV infection.
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48 274 *EGFR facilitates HEV infection independent of its kinase activity*
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51 275 To gain additional insight whether EGFR kinase activity and signaling are relevant for HEV infection
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53 276 and entry, we stably expressed the EGFR mutants EGFR-L858R and EGFR-K745A in HepG2 cells.
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55 277 While a mutation of leucine at the position 858 to arginine leads to a constitutive activation of EGFR
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57 278 kinase, the mutation of lysine 745 to alanine impairs its kinase function (see Fig. 5A)^{33,34}. After
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59 279 confirmation of the ectopic expression of EGFR and its mutants via western blot and
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3 280 immunofluorescence staining (Fig. 5B–C upper panels), the activity levels of the EGFR kinase domains
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5 281 were evaluated (Fig. 5C middle panel). Therefore, the respective cells were serum starved overnight
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7 282 followed by addition of EGF (16.5 nM or 100 ng/mL) for 15 min. An immunofluorescence staining was
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9 283 performed to determine the phosphorylation status at Tyr1068. While only few pEGFR(1068) signals
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11 284 were observed in the cytoplasm of empty vector cells and cells ectopically expressing the EGFR-K745A
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13 285 mutant, a high abundance of pEGFR(1068) signals was observed in EGFR and EGFR-L858R
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15 286 expressing cells (Fig. 5C middle panel). To demonstrate the ability of the EGFR-L858R mutant to
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17 287 signal even in the absence of ligand binding, we performed immunofluorescence analysis of EGFR's
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19 288 phosphorylation status at Tyr1068 after overnight serum starvation. High abundance of pEGFR(-1068)
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21 289 signals were visible even without ligand induction in EGFR-L858R HepG2 cells, while only very low
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23 290 levels of pEGFR(-1068) signals were visible in EGFR-WT cells (supplementary Fig. S6). Hereby, we
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25 291 validated the constitutively active kinase function of EGFR in EGFR-L858R HepG2 cells, and its
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27 292 inactivation in EGFR-K745A HepG2 cells.

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31 293 We next tested whether the different EGFR mutants were capable to facilitate HEV infection compared
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33 294 to EGFR wildtype (EGFR-WT). To this end, the respective cell lines were infected with HEV and the
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35 295 HEV infection quantified via immunofluorescence staining. Similar to the EGFR-WT, we detected
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37 296 significantly increased HEV infection in the presence of both EGFR-L858R and EGFR-K745A,
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39 297 compared to empty vector control (Fig. 5B and C lower panels), implying that EGFR facilitates HEV
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41 298 infection independent of its kinase activity. Furthermore, we observed no effect on HEV RNA
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43 299 replication nor on HEV progeny virus production after electroporation of HEV IVT RNA in the
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45 300 presence of the different EGFR mutants (supplementary Fig. S7A–B). In summary, our obtained results
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47 301 suggest that access to EGFR is critical for HEV and facilitates infection independent of its kinase
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49 302 activity.

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53 303 In order to analyze whether extracellular EGFR interaction plays a role in HEV infection, we utilized
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55 304 an EGFR mutant lacking its ligand binding domain (EGFRvIII), while obtaining constitutively low
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57 305 levels of active signaling³⁵. We first validated the ectopic expression of EGFR-WT and EGFRvIII
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59 306 mutant in HepG2 cells via western blot and immunofluorescence (Fig. 5D and E upper panels).

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3 307 Subsequently, we tested whether the EGFRvIII mutant was capable to facilitate HEV infection by
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5 308 infecting the respective cells with HEVcc (p6) and quantification of HEV infection via
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7 309 immunofluorescence. Of note, we observed a significant increase of HEV infection in the presence of
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9 310 EGFR-WT but not in the presence of EGFRvIII compared with empty vector cells suggesting that the
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11 311 lack of the EGF binding domain abolishes the EGFR-mediated proviral effect. Further, we detected no
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13 312 effect on HEV RNA replication nor on HEV progeny virus production after electroporation of HEV
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15 313 IVT RNA in the presence of the different EGFR mutants (supplementary Fig. S7C–D). Taken together,
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17 314 the obtained data imply that the EGFR ligand binding domain plays a crucial role in facilitating HEV
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19 315 infection.

22 316 *EGFR is critical for HEV entry in HepaRG cells and PHHs*

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25 317 To further validate our findings in a cell culture model exhibiting more characteristics of hepatocytes
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27 318 *in vivo*, differentiated HepaRG cells were used. HepaRG cells exhibit many key metabolic enzymes and
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29 319 receptors that make them an attractive alternative model for *in vitro* studies and as such have already
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31 320 been extensively used in the study of other hepatotropic viruses^{36,37}. Therefore, HepaRG cells were
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33 321 differentiated into cholangiocyte- and hepatocyte-like cells²⁹. Successful differentiation was confirmed
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35 322 via immunofluorescence staining for the hepatocyte marker albumin (Fig. 6A). To address the
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37 323 importance of EGFR in this cell culture system, we infected differentiated HepaRG cells with HEVcc
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39 324 (p6) in the presence of EGFR modulators and subsequently determined ORF2+ cells (Fig. 6B left and
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41 325 C upper panel). We detected that Erlotinib efficiently reduced HEV infection. In contrast to our previous
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43 326 findings in hepatoblastoma cells, EGF increased HEV infection.

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47 327 To evaluate whether EGFR modulators are capable to restrict HEV entry in primary cells, we pretreated
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49 328 PHHs with EGFR modulators followed by HEVcc (p6) infection. Both, Erlotinib and Cetuximab,
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51 329 reduced HEV infection by approximately 61% and 55%, respectively, when compared to untreated cells
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53 330 (Fig. 6B right and C lower panel), highlighting the potential restriction capacity of EGFR modulators
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55 331 during HEV infection *ex vivo*. Taken together, these data suggest that EGFR is critical for HEV entry
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57 332 in primary cells and that HEV infection can be restricted by application of EGFR modulators during
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59 333 HEV inoculation.

334 Discussion

335 Although HEV is an increasing health-burden, knowledge of HEV's pathogenesis and life cycle has
336 been scarce so far. Despite the fact that HEV entry is an appealing target for pharmacological
337 intervention, druggable host factors to prevent HEV entry have yet to be identified^{12,13}. EGFR is a
338 receptor tyrosine kinase and as such involved in cell migration, proliferation and differentiation^{20,21}.
339 Importantly, EGFR has been found to be a host factor for numerous viruses affecting different life cycle
340 steps. Viruses like severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)³⁸ and Epstein-Barr
341 virus (EBV)³⁹ among others regulate EGFR expression and recycling, thereby isolating host cells from
342 host-specific signals forcing them to respond solely to viral signals and thus optimizing cellular
343 environments for productive infections. Other viruses, including Influenza A virus (IAV)⁴⁰,
344 rhinoviruses and respiratory syncytial virus (RSV)⁴¹, manipulate EGFR signaling to antagonize viral
345 inflammation and host anti-viral systems. Furthermore, EGFR signaling is utilized for viral entry and
346 replication by remodeling the actin network enabling entry (i.e. human cytomegalovirus [HCM]⁴²,
347 Herpes Simplex virus 1 [HSV-1]⁴³), or inducing favorable environments for replication, (i.e. EBV⁴⁴).
348 Additionally, EGFR's trafficking is exploited by HCV¹⁸ and hepatitis B virus HBV⁴⁵, thereby
349 facilitating cell entry by linking the virus-host cell complex to the endocytic machinery. Finally, EGFR
350 can act as a co-receptor stabilizing virus-host cell complexes or enriching initial or sequential receptors.
351 In this study, we present EGFR as a new host factor for HEV in human hepatocytes.

352 First of all, we found that endogenous EGFR is abundantly expressed in hepatocytes and cholangiocytes
353 in human liver *in vivo* (Fig. 1) and plays a role in HEV infections using EGFR-specific siRNA and the
354 EGFR kinase inhibitor Erlotinib (Fig. 2). We were able to confirm EGFR's role in iPSC-derived HLCs
355 via shRNA knockdown. Furthermore, different strains of HEV (p6 and 83-2), as well as non-enveloped
356 and enveloped HEV were significantly affected by EGFR inhibition, albeit to slightly different degrees.
357 Our findings, that ectopic expression of EGFR (Fig. 4) increases HEV infections further implies that
358 EGFR is critical for HEV. To dissect the effect of EGFR in the HEV life cycle, we performed assays
359 specific for each step (Fig. 3). Here, we were able to show that the effect is specific for the entry process,
360 while leaving the attachment, replication and assembly unaltered. We further analyzed the impact of

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3 361 EGFR kinase activity on HEV infectivity, using a constitutively active kinase mutant and a kinase-dead
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5 362 mutant. ~~Despite T~~the expression of ~~both the two EGFR~~ mutants, ~~the -constitutively active and the~~
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7 363 ~~kinase-dead mutant, both~~ facilitating HEV infections; ~~which implies that an effect of the different~~
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9 364 kinase function ~~does not affect the HEV life cycle. is not indicated here~~. However, by deprivation of
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11 365 EGFR's ligand binding domain (EGFRvIII mutant) the proviral effect of EGFR is lost, underlining the
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13 366 crucial role of its ligand binding domain to HEV infection. We therefore suggest that the receptor itself
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15 367 or non-canonical pathways modulate HEV entry. At this stage of understanding, two possible
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17 368 mechanisms are likely for EGFR's effect on HEV entry: (1) via utilizing EGFR's trafficking or (2) via
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19 369 EGFR as a co-receptor. For example, EGFR endocytosis and trafficking are hijacked by HBV⁴⁵. There,
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21 370 the EGFR endocytic machinery drives the translocation of HBV-receptor (NTCP)-bound HBV from
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23 371 the cell surface through the endosomal network to late endosomes and lysosomes thus providing an
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25 372 entry mechanism. However, this mechanism is rather unlikely to be the reason for EGFR's effect on
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27 373 HEV entry, since the EGFR modulators and EGFR mutants modulate EGFR trafficking in a different
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29 374 manner but show similar effects⁴⁶⁻⁴⁹ and HEV has been found to be internalized depending on clathrin-
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31 375 mediated endocytosis¹⁵. For example, while Erlotinib and Cetuximab have been found to induce
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33 376 caveolin-mediated internalization of EGFR, low concentrations (<2 ng/mL) EGF activate EGFR
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35 377 endocytosis in a clathrin-dependent manner. Even in the presence of higher concentrations (100 ng/mL,
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37 378 16.5 nM) of EGF roughly 60% of the receptor have been reported to be endocytosed clathrin-
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39 379 mediated⁴⁶⁻⁴⁹. Given the differences in the internalization routes of different EGFR variants and the
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41 380 crucial role of EGFR's extracellular ligand-binding domain, ~~as well as the proposed distinct entry~~
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43 381 ~~mechanisms for enveloped and non-enveloped HEV~~⁵⁰, we speculate a mechanism that involves EGFR
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45 382 as an entry co-factor. EGFR could either increase the binding avidity to an initial yet unknown receptor
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47 383 or sequential proteins important for ~~the entry of enveloped and non-enveloped HEV's entry~~.
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49 384 Alternatively, EGFR could associate to or stabilize an initial receptor or sequential proteins.
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51 385 Furthermore, enrichment of an initial receptors important for HEV's entry could be a possible
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53 386 mechanism of EGFR as a co-factor as well. EGFR modulators might lower the surface expression of
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55 387 EGFR or disrupt associations of EGFR with entry receptor(s) of HEV thus decreasing HEV infection⁴⁶.
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57 388 For instance, the EGFR kinase inhibitor Erlotinib impacts EGFR's localization and other non-signaling
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3 389 pathways in addition to inhibiting its classical signaling, which causes EGFR to be arrested inside the
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5 390 cell and degraded⁴⁶. Further, EGF can induce EGFR activation and internalization, reducing the surface
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7 391 level of EGFR temporarily^{24,25}. However, proving the proposed mechanisms is not possible yet, as the
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9 392 main receptor needed for HEV entry has not been identified so far and knowledge on its entry process
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11 393 is scarce^{12,13}, thus limiting available assays.

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14 394 In conclusion, our study revealed EGFR as a novel host factor for HEV's entry process in
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16 395 hepatoblastoma-cell culture systems and also *ex vivo* in PHHs, underlining the relevance of this
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18 396 particular factor. EGFR's kinase function and canonical signaling has been found to be of no influence,
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20 397 while EGFR's ligand-binding domain was found to be crucial for the facilitation of HEV infection.
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22 398 Therefore, EGFR is likely an entry co-factor either increasing the binding avidity, stabilizing or
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24 399 associating with initial HEV receptors (or sequential proteins) thus bringing the initial receptor (or
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26 400 sequential proteins) in close proximity. Alternatively, EGFR could augment necessary receptors and
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28 401 thus facilitating viral entry. However, future studies have to address the identification of the initial
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30 402 receptor(s) in order to implement novel assays studying the receptor's specific role. Importantly, EGFR
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32 403 has been identified to play a diverse range of roles in viral infection, its participation in HEV infections
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34 404 therefore broadens its scope and gives not only great advances in the understanding of EGFR's role in
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36 405 viral infections but also substantially expands the scarce knowledge of HEV host factors.
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43
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50 411 T.L.M., A.-K.M., R.M.F., P.B. and V.K. performed research, D.D., R.B. and F.W.R.V. contributed
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52 412 reagents, J.A.S., A.G., D.T., V.K. and E.S. analyzed data; J.A.S. wrote the original draft; J.A.S.,
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54 413 T.L.B., Y.B., A.G., T.L.M., P.B., D.D., D.T., V.K. and E.S. reviewed and edited the original draft.
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3 581 **Figure Legends**
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6 582 **Fig.1: EGFR is expressed endogenously in primary human liver cells.** (A) T-distributed stochastic
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8 583 neighbor-embedding (t-SNE) plots highlighting mRNA expression of ALB and EGFR across all cells
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10 584 of healthy human liver tissue³². The color of each cell represents the gene expression according to the
11
12 585 corresponding legend as log₂ value of the expression. Cell type annotation was transferred from
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14 586 Aizarani et al.³² (B) Violin plot showing the normalized expression of proposed HEV host factors
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16 587 EGFR, TSG101 and Rab5. The color code depicts the different cell types present in the dataset.
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22 589 **Fig. 2: Endogenous EGFR is critical for HEV infection.** (A) EGFR protein expression in HepG2
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24 590 cells 48 h post transfection with EGFR-specific siRNAs or non-targeting control siRNA (siCtrl)
25
26 591 analyzed via western blot (left) and immunofluorescence (right). (B) HEVcc (p6) infection in HepG2
27
28 592 cells transfected with EGFR-specific siRNAs and control siRNA. Cells were infected with HEVcc (p6)
29
30 593 two days post transfection and FFU/well determined after fixation 5 d p.i. Left: Quantification of focus
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32 594 forming units (FFU) of the full well normalized to cells transfected with control siRNA. Right:
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34 595 representative immunofluorescence images stained for ORF2 protein. (C, D) HEVcc p6 non-enveloped
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36 596 and enveloped, as well as 83-2 non-enveloped infection in HepG2 cells under treatment of EGFR
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38 597 modulators Erlotinib (33 μM, Erlo), EGF (16.5 nM) and Cetuximab (34 nM, Cetu) compared to
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40 598 untreated control cells (UTC), while the HEV inhibitor Ribavirin (50 μM, Rbv) served as control.
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42 599 (C) Representative immunofluorescence images stained for ORF2 protein in HEVcc (p6) infected
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44 600 HepG2 cells under EGFR modulator treatment. (D) FFUs/well were counted in HEVcc p6 non-
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46 601 enveloped (left), p6 enveloped (middle) or 83-2 (right) infected HepG2 cells under EGFR modulator
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48 602 treatment and normalized to UTC. To test significance of mean differences, student t-test (B) and one-
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50 603 way ANOVA followed by Dunnett's multiple comparison test (D) were used, p values < 0.05 (*), <0.01
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52 604 (**), <0.001 (***) and <0.0001 (****), p values >0.05 were considered to be non-significant (ns). All
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54 605 infection experiments were performed in triplicates. Mean and SEM are depicted from at least three
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56 606 independent experiments. Scalebars = 100 μm.
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4 607 **Fig. 3: EGFR has no effect on attachment, replication or assembly but affects the entry process**
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6 608 **of HEV. (A)** Quantification of HEVcc (p6) entry in HepG2 cells under EGFR modulator treatment
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8 609 (Erlotinib (33 μ M, Erlo), EGF (16.5 nM) and Cetuximab (34 nM, Cetu) compared to untreated control
9
10 610 cells (UTC). Cells were pre-treated for 30 min with EGFR modulators prior to infection with HEVcc
11
12 611 (p6) for 8 h under modulator treatment, following a medium change and 5 d infection in medium
13
14 612 without virus or treatment. 50 μ M of Rbv was renewed after the medium change 8 h p.i., hereby serving
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16 613 as control of efficient inhibition. **(B)** Quantification of HEVcc (p6) attachment under EGFR modulator
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18 614 treatment in FFU/well in HepG2/C3A cells. Cells were pretreated with EGFR modulators for 30 min at
19
20 615 37 °C before addition of virus for 2 h on ice, allowing attachment but not entry. HEV inhibitor Rbv
21
22 616 served as negative control here and anti-HEV serum (1:200) as positive control neutralizing HEVcc
23
24 617 (p6). Cells were washed thrice before incubation without additives for 5 d p.i.. **(C)** Quantification of
25
26 618 effects of EGFR modulators on HEVcc post-attachment steps in ORF2 protein positive cells per well
27
28 619 in HepG2/C3A cells. Cells were incubated on ice for 30 min before infection with HEVcc (p6) on ice
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30 620 for 2 h. Inoculum was removed and modulator added to the cells for the indicated time (8 h or 3 d)
31
32 621 allowing modulation only during post-attachment processes. **(D)** HEV p6 replication levels in RNA
33
34 622 subgenomic replicon (SGR) system at 72 h p.e. in HepG2 cells under modulator treatment. **(E)** Viral
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36 623 titers of HEVcc (p6) produced after electroporation of HEV Kernow-p6 RNA into HepG2 cells
37
38 624 following virus production of HEVcc (p6) under modulator treatment, thereby excluding the HEV
39
40 625 entry. Titration of progeny virus on HepG2/C3A cells.
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42
43 626 To test significance of mean differences, one-way ANOVA followed by Dunnett's multiple comparison
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45 627 test was used, p values < 0.05 (*), <0.01 (**), <0.001 (***) and <0.0001 (****), p values >0.05 were
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47 628 considered to be non-significant (ns). All assays were performed in triplicates. Mean and SEM are
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49 629 depicted from three independent experiments.

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54 631 **Fig. 4: Ectopic EGFR expression facilitates HEV infection and is sensitive to EGFR modulators**
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56 632 **affecting the HEV entry process. (A)** EGFR protein expression in HepG2 cells stably expressing
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58 633 EGFR (HepG2-EGFR) analyzed via western blot (left) and immunofluorescence (right). Cells
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3 634 transduced with an empty vector served as control. **(B)** HEVcc (p6) infection in HepG2-EGFR cells.
4
5 635 Left: Quantification of focus forming units (FFU) of the full well normalized to empty vector expressing
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7 636 cells- Right: Representative immunofluorescence images stained for ORF2 protein. **(C)** Right: Entry
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9 637 assay of HEVcc (p6) infection in HepG2-EGFR under EGFR modulator treatment (Erlotinib (33 μ M,
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11 638 Erlo), EGF (16.5 nM) and Cetuximab (34 nM, Cetu) compared to untreated control cells (UTC), while
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13 639 the HEV inhibitor Ribavirin (50 μ M, Rbv) served as control for efficient inhibition. Cells were pre-
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15 640 treatment for 30 min with EGFR modulators prior to infection with HEVcc (p6) for 8 h under modulator
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17 641 treatment, following a medium change and 5 days infection in medium without virus or treatment. Rbv
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19 642 (50 μ M) was replenished as it served as control for efficient inhibition. Dashed line indicates level of
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21 643 normalized FFUs/well of untreated empty vector cells. Middle: HEV (p6) replication level in RNA
22
23 644 subgenomic replicon (SGR) system 72 h p.e in HepG2-EGFR cells normalized to relative light unit
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25 645 (RLU) levels at 4 h p.e.. Right: HepG2-EGFR cells transfected with HEV Kernow-p6 RNA for virus
26
27 646 production. Virus titers determined from non-enveloped virus produced in HepG2-EGFR cells.
28
29 647 **(D)** Representative immunofluorescence images stained for ORF2 protein in HEVcc (p6) infected
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31 648 HepG2-EGFR cells under EGFR modulator treatment during entry, corresponding to the left panel of
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33 649 **(C)**. To test significance of mean differences, student t-test (B and C middle and right) and one-way
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35 650 ANOVA followed by Dunnett's multiple comparison test (C left panel) were used, p values < 0.05 (*),
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37 651 <0.01 (**), <0.001 (***) and <0.0001 (****), p values >0.05 were considered to be non-significant
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39 652 (ns). All infection experiments were performed in triplicates. Mean and SEM are depicted from at least
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41 653 three independent experiments. Scalebars = 100 μ m.
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655 **Fig. 5: Ectopic expression of EGFR mutants indicates no effect of EGFR signaling in HEV**
656 **infection.** **(A)** Schematic diagram of EGFR (-mutant) domains. JM= Juxtamembrane domain; TM=
657 Transmembrane domain; KD= Kinase domain; RD= Regulatory domain. **(B)** EGFR protein expression
658 in HepG2 cells ectopically expressing EGFR (-mutants) analyzed via western blot (upper). HEVcc (p6)
659 infection in EGFR (-mutant) ectopically expressing HepG2 cells (lower). Quantification of FFUs/well
660 normalized to cells stably expressing only the empty vector **(C)** Immunofluorescence analysis of EGFR

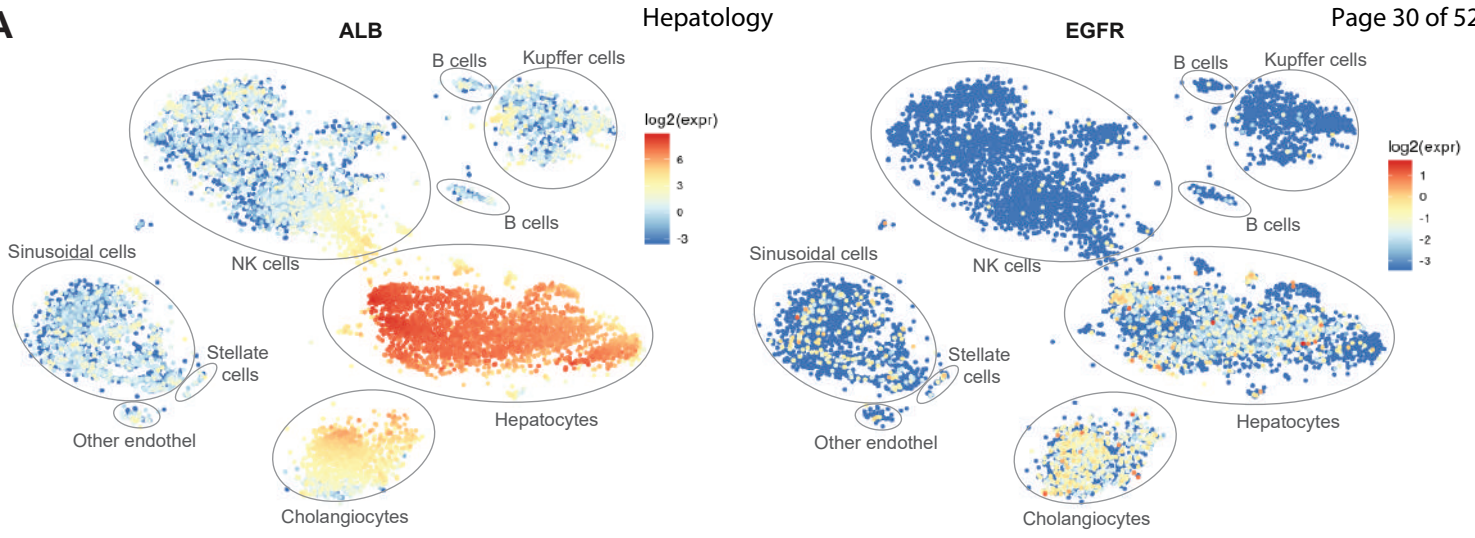
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3 661 protein expression (upper) and EGFR phosphorylation at Tyr1068 after overnight FCS starvation of
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5 662 EGFR (-mutant) ectopically expressing HepG2 cells and challenge with EGF (16.5 nM) for 15 min
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7 663 (middle). Representative fluorescence images of HEVcc (p6) infection in EGFR (-mutant) expressing
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9 664 cells after staining against ORF2 protein (lower). (D) EGFR protein expression HepG2 cells ectopically
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11 665 expressing the EGFR WT or EGFR vIII mutant analyzed via western blot (upper). HEVcc (p6) infection
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13 666 in EGFR (-mutant) ectopically expressing HepG2 cells (lower). Quantification of FFUs/well
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15 667 normalized to cells stably expressing only the empty vector. (E) Immunofluorescence analysis of EGFR
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17 668 protein expression (upper) and representative fluorescence images of HEVcc (p6) infection in EGFR (-
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19 669 mutant) expressing cells after staining against ORF2 protein (lower). Infection experiments were
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21 670 performed in triplicates. To test significance of mean differences, one-way ANOVA followed by
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23 671 Dunnett's multiple comparison test was used, p values < 0.05 (*), <0.01 (**), <0.001 (***) and <0.0001
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25 672 (****), p values >0.05 were considered to be non-significant (ns). Mean and SEM are depicted from
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27 673 three independent experiments. Scalebars = 100 μ m.
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33 675 **Fig. 6: The critical entry effect of EGFR was verified in HepaRG cells and primary human**
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35 676 **hepatocytes (PHHs).** (A) Phase contrast and immunofluorescence images of HepaRG cells during
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37 677 differentiation. Yellow arrow indicates hepatocyte-like cells, red arrow cholangiocyte-like cells.
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39 678 (B, C) HEVcc (p6) infection in differentiated HepaRG cells under EGFR modulator treatment during
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41 679 the full infection time of 5 days (Erlotinib (33 μ M, Erlo), EGF (16.5 nM) and Cetuximab (34 nM, Cetu)
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43 680 compared to untreated control cells (UTC), while 25 μ M of the HEV inhibitor Ribavirin (Rbv) served
44
45 681 as control). (B) Quantification of ORF2-positive cells via CellProfiler analysis in percent of all counted
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47 682 DAPI nuclei per image and (C) representative fluorescence images stained against ORF2 protein.
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49 683 (D, E) HEVcc (p6) infection in PHHs under EGFR modulator treatment during the entry of HEV,
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51 684 meaning a 30 min pre-treatment with EGFR modulators prior to infection with HEVcc (p6) for 16 h
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53 685 under modulator treatment, following 3 days incubation time without inoculum or modulators.
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55 686 (D) Quantification of ORF2-positive cells via CellProfiler analysis in percent of all counted DAPI
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57 687 nuclei per image and (E) representative fluorescence images stained against ORF2 protein. Infection
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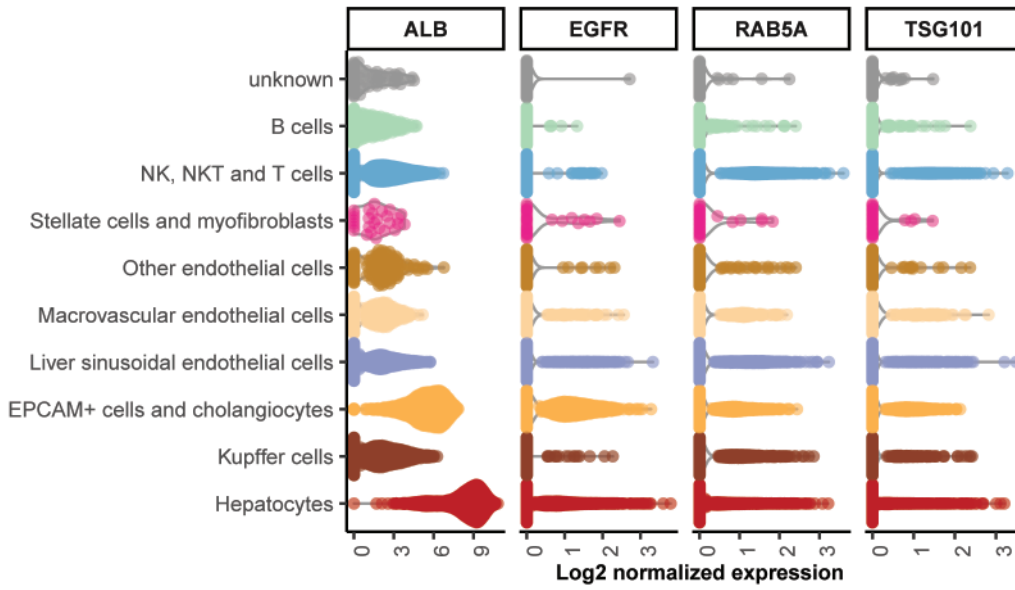
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3 688 experiments were performed in duplicates with at least ten images taken for analysis per experiment.
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5 689 To test significance of mean differences, one-way ANOVA followed by Dunnett's multiple comparison
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7 690 test (D) was used, p values < 0.05 (*), <0.01 (**), <0.001 (***) and <0.0001 (****), p values >0.05
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9 691 were considered to be non-significant (ns). Mean and SEM are depicted from two (HepaRG) or four
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11 692 (PHH) independent experiments. Scalebars = 100 μ m.
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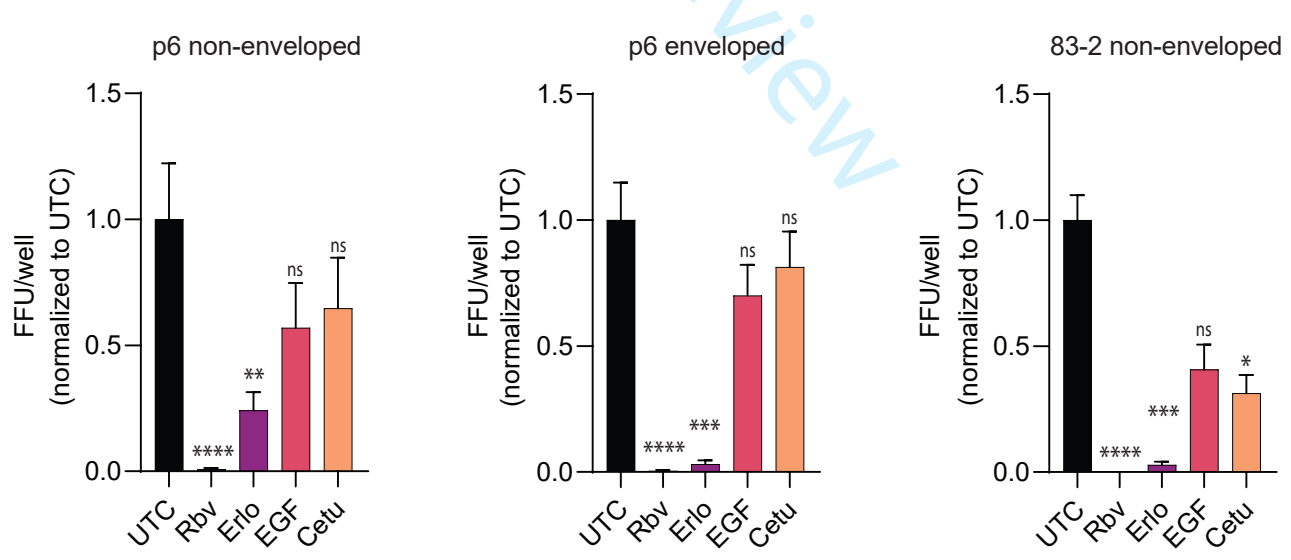
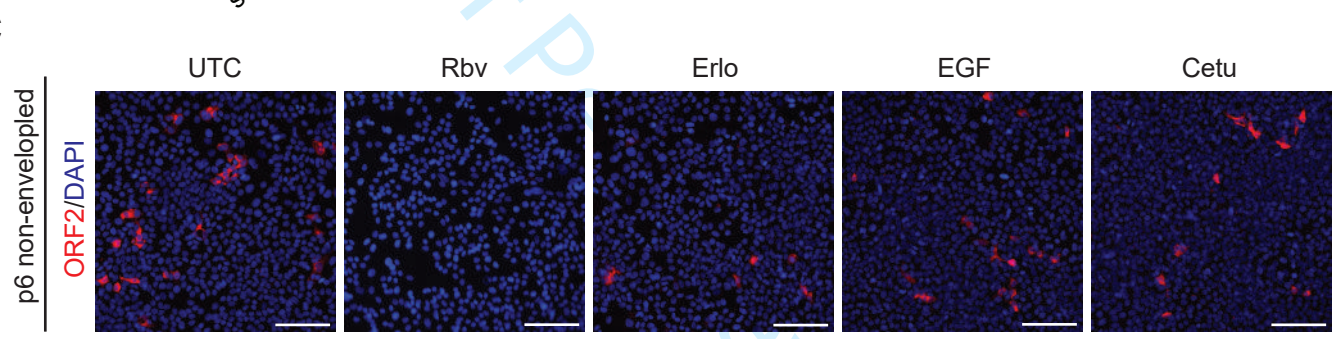
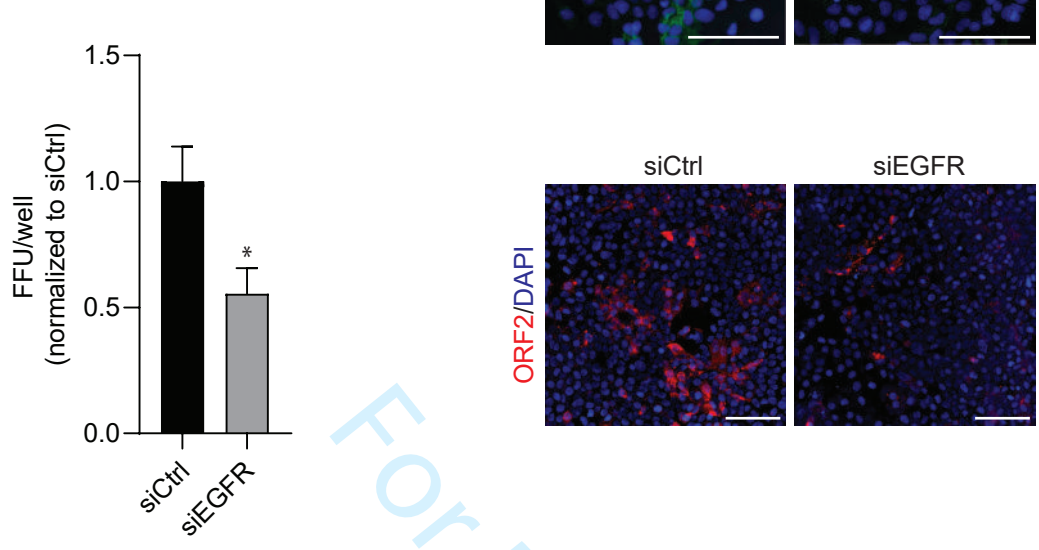
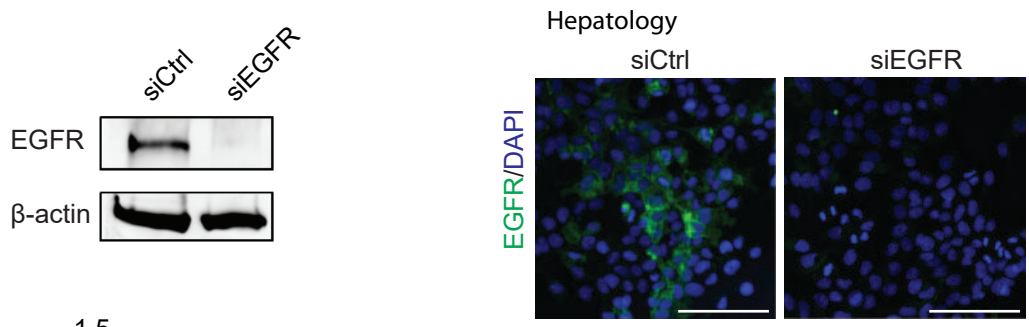
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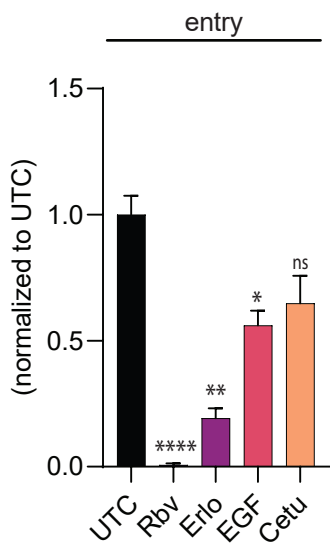
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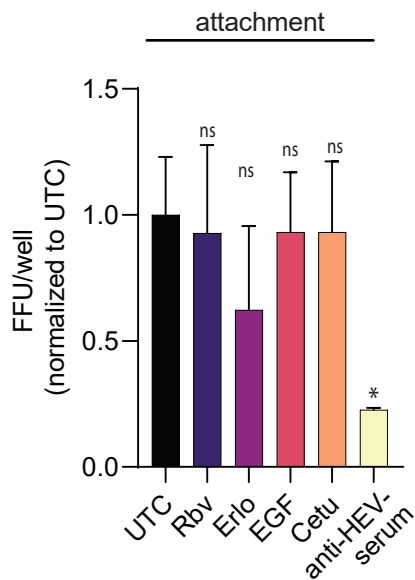
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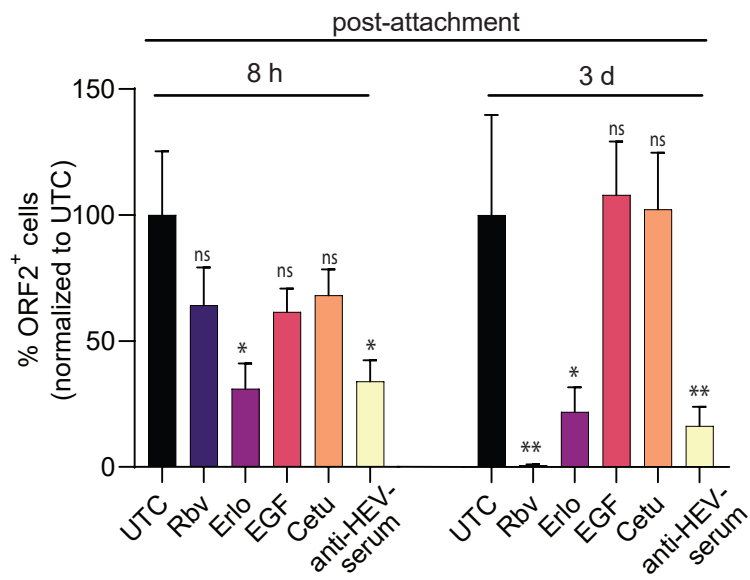
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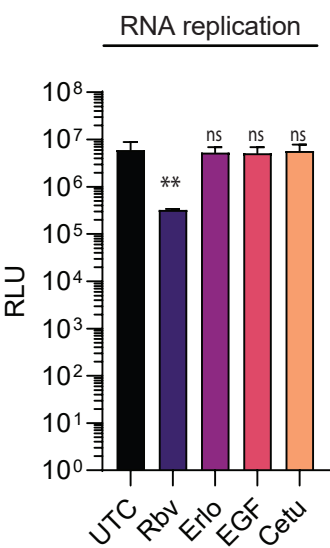
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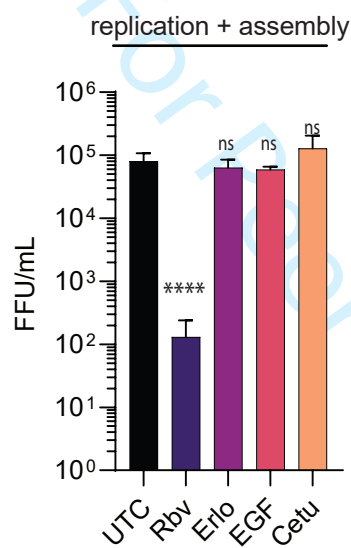
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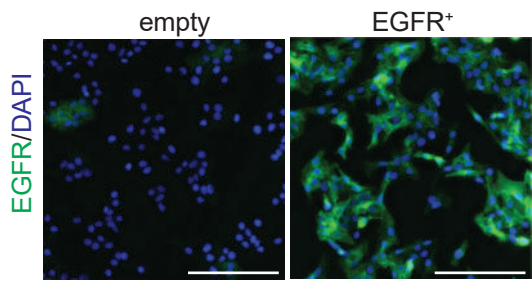
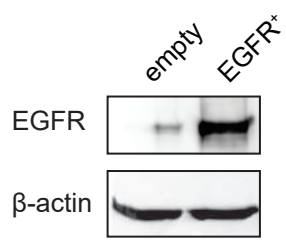
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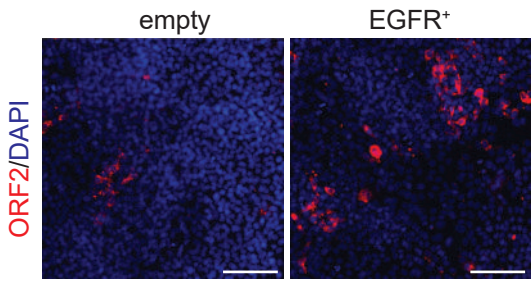
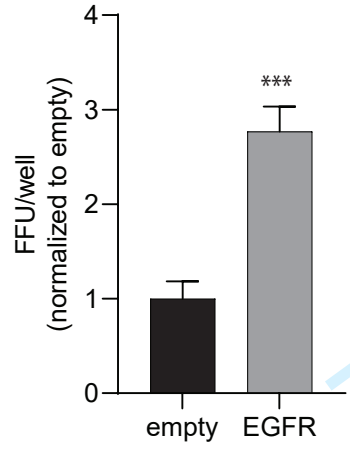
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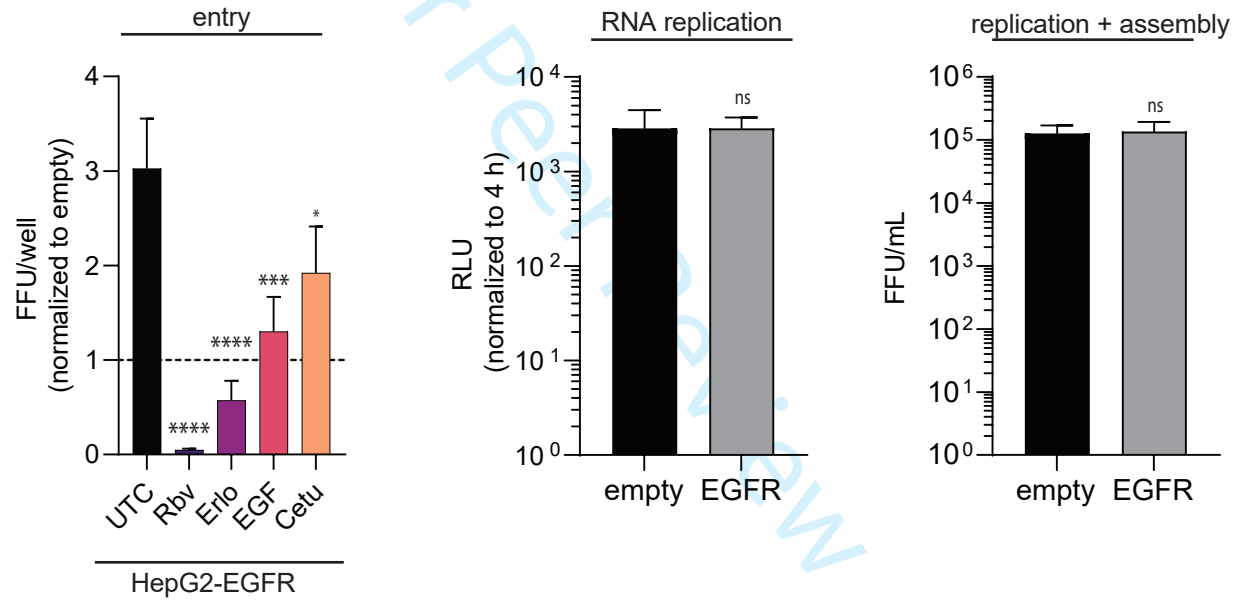
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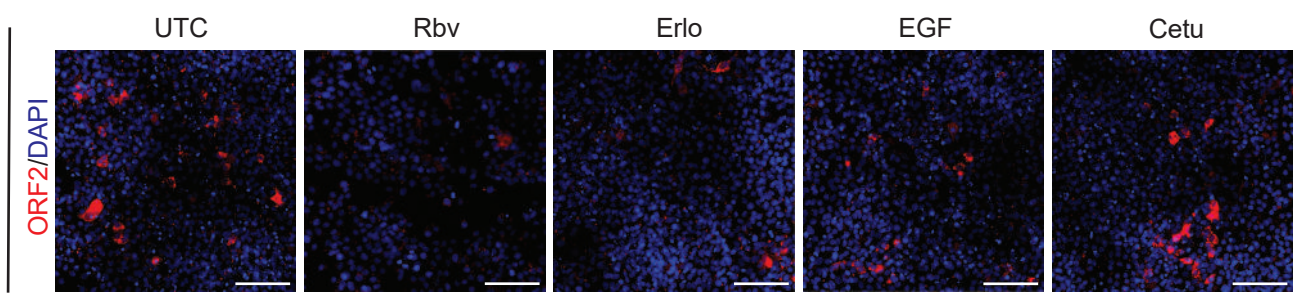
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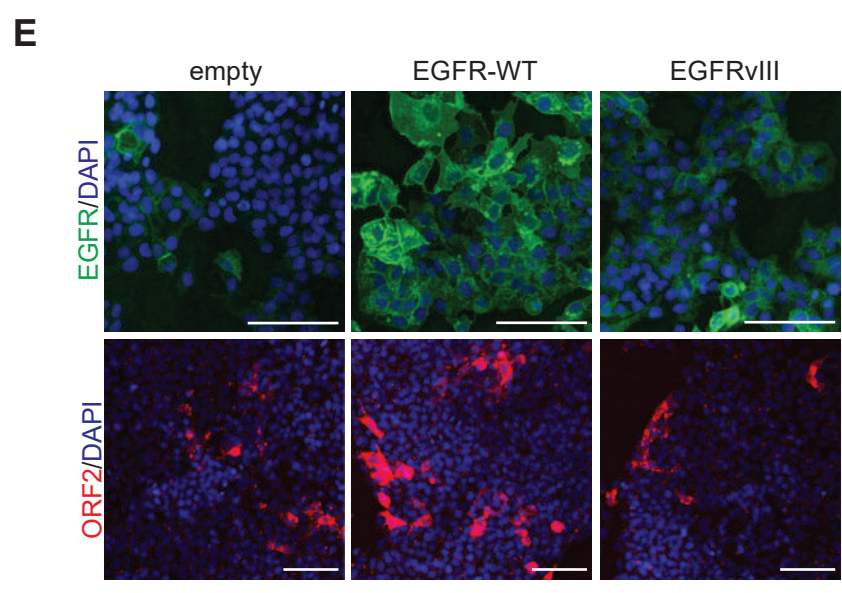
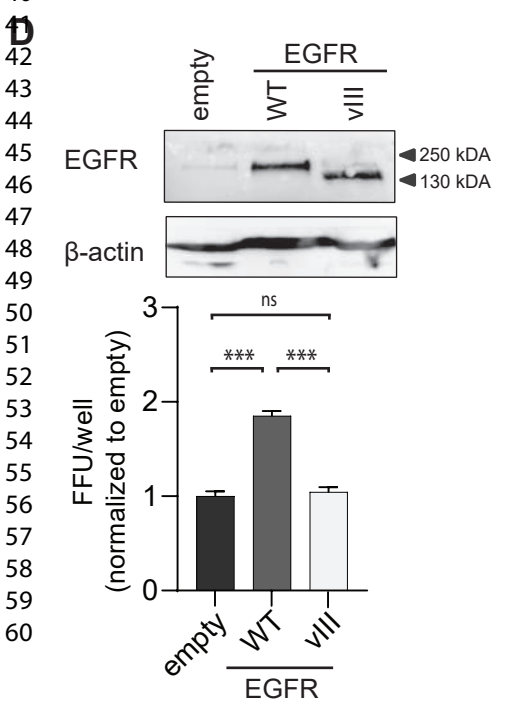
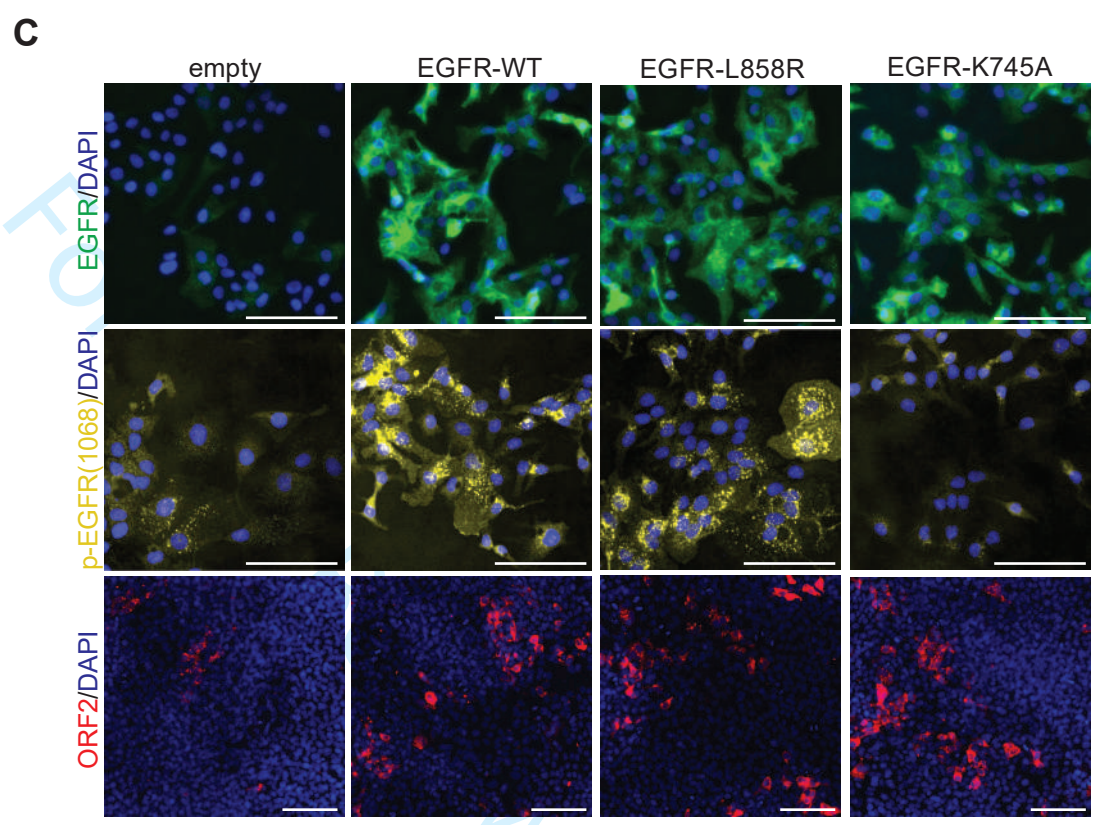
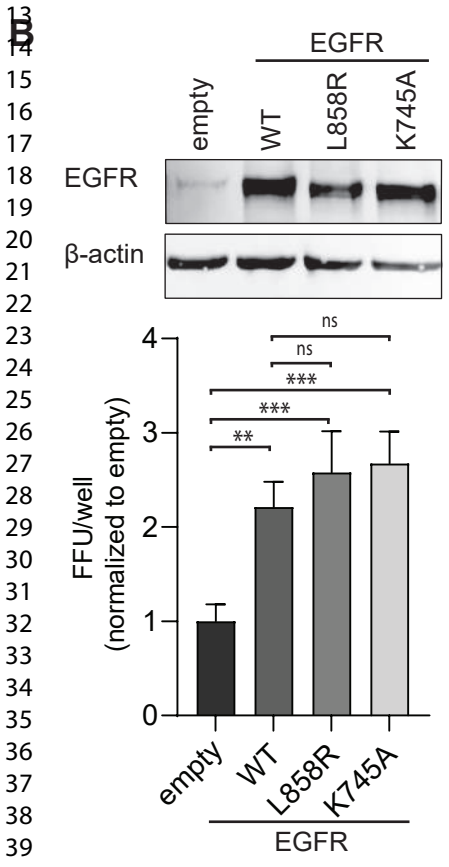
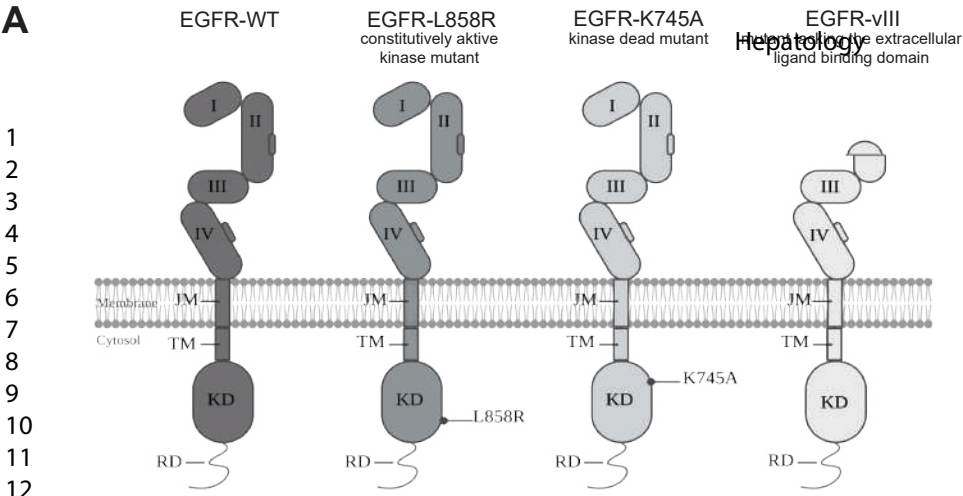


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3 **1 Supplementary Information – HEP-22-1113**

4 **2 Epidermal Growth Factor Receptor Modulates Hepatitis E Virus Entry in Human Hepatocytes**

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32 **Material and Methods**

33 *Hepatocyte-Like Cells (HLCs)*

34 Hepatocyte-like cells were made from induced pluripotent stem cells (iPSCs) as described previously¹
35 and plated for differentiation on a 48-well plate.

37 *Production of Ectopically Expressing Cell Lines via Lentiviral Transduction*

38 For the production of lentiviral particles, 8×10^5 293T cells were seeded on collagen-coated 6-well
39 plates. The following day, the 293T cells were transfected with the plasmids pcz-VSV-G, pCMV-
40 dR8.74 and pWPI-BLR with the respective gene of interest using Lipofectamine 2000 (Invitrogen, Cat.
41 Nr. 11668019) following the manufacturer's instructions. Six hours post transfection, a medium change
42 was done and the lentiviral particles were harvested 48 h and 72 h post transfection by collecting the
43 supernatant and filtering it through a 0.45 μm mesh (Filtropur 0.45, Sarstedt, Cat. Nr. 83.1826) to
44 remove any cell debris. 1×10^5 target cells (HepG2) per well were seeded on a 6-well plate prior to
45 treatment with 4 $\mu\text{g}/\text{mL}$ polybrene (Sigma-Aldrich, Cat. Nr. TR-1003) and infection with 1 mL of
46 lentiviral particles for 6–8 h. Selection of the transduced cells was started 48 h post transduction using
47 5 $\mu\text{g}/\text{mL}$ blasticidin-S-hydrochlorid (Fisher Bioreagents, Cat. Nr. BP2647) and further maintained in
48 DMEM complete containing blasticidin-S-hydrochlorid. Validation of the ectopic expression was done
49 via western blot and immunofluorescence staining.

51 *siRNA-Mediated Knock Down*

52 Knock down of endogenously expressed EGFR was performed using two validated short interfering
53 RNAs (siRNA, Ambion, ID s564 and s565, Cat. Nr. 4427038) and a control siRNA (Ambion, Silencer
54 Select, Cat. Nr. 4390843). 0.5 μM each of the siRNA were transfected using Lipofectamine RNAiMax
55 (Invitrogen, Cat. Nr. 13778) following the manufacturer's instructions. Two days post transfection, the
56 cells were either subjected to infection assays or lysed for western blot analysis.

59 *shRNA-Induced Knock Down via Adeno-Associated Viral (AAV) Transduction*

60 The sequence of the shRNA targeting EGFR was obtained from Rothenberg *et al.*²(sequence of
61 shEGFR: GCTGGATGATAGACGCAGATA, sequence of shCtrl:
62 GGTCGTGAACTAATCAGAGGA). The forward strand oligonucleotides were designed by starting
63 with a 5' CACC overhang for cloning using BsmBI, followed by the sense sequence, a connecting 7 nt
64 loop (TCAAGAG) sequence and finally the antisense sequence (targeting sequence). The reverse strand
65 was designed using a 5' AAAA overhang. The shRNA genes were cloned into a self-complementary
66 AAV6 vector under the U6 promoter. Iodixanol purified and recombinant AAVs were produced as
67 described previously³. HLCs were transduced with AAVs three days prior to HEV infection. The
68 inoculum was removed 24 h post transduction. The cells washed once with PBS and fresh HLC culture
69 medium renewed.

71 *In Vitro Transcription and Electroporation*

72 Before *in vitro* transcription, HEV Kernow-C1-p6-full length and -Gluc plasmid were linearized using
73 MluI (New England Biolabs, Cat. Nr. R3198), while HEV 83-2-containing plasmids were linearized
74 with HindIII (New England Biolabs, Cat. Nr. R3104). *In vitro* transcribed RNA (IVTs) were produced
75 as described in Todt *et al.*⁴. IVTs were subsequently transfected into the respective cells using the
76 electroporation technique described in⁴. Shortly, 5 µg of *in vitro* transcribed RNA were mixed with
77 5×10^6 cells in 400 µL cytomix containing 2 mM ATP (Cayman Chemical, Cat. Nr. 14498) and 5 mM
78 glutathione (Sigma Aldrich, Cat. Nr. # G4251). After electroporation using the Gene Pulser system
79 (Bio-Rad), cells were immediately transferred to 12.1 mL of DMEM complete and the cell suspension
80 was seeded in respective plates depending on the experiment (2×10^4 cells/well seeded in a 96-well plate
81 for luciferase assays, 12.5 mL seeded in a 10 cm dish for virus production).

83 *Production of Cell Culture-Derived HEV Particles (HEV_{CC})*

84 HEV_{CC} (p6 and 83-2) was produced as previously described⁴. In brief, HEV IVTs were electroporated
85 into HepG2 cells. Seven days post electroporation, the supernatant containing enveloped HEV_{CC} was
86 filtered through a 0.45 µm mesh (Filtropur 0.45, Sarstedt, Cat. Nr. 83.1826) and stored at 4 °C for up

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3 87 to 7 days. To harvest non-enveloped HEVcc, the cells were trypsinized, resuspended in fresh DMEM
4
5 88 complete and lysed via three freeze-thaw cycles in liquid nitrogen. The lysate was cleared from cell
6
7 89 debris by a 10 000×g centrifugation for 10 min and titrated on HepG2/C3A cells to determine viral
8
9 90 titers. Non-enveloped HEVcc was frozen at -80°C until further usage. Cells were fixed with 3%
10
11 91 paraformaldehyde (PFA, Roth, Cat. Nr. 93351) seven days post infection for immunofluorescence
12
13 92 staining against ORF2 protein and determination of the number of focus forming units (FFU) according
14
15 93 to Todt et al. ⁴.

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19 20 95 *HEV Infection Assays with Non-Enveloped HEVcc*

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22 96 For infection assays, either 3.5×10^3 HepG2(-derived) cells were seeded on collagen-coated 96-well
23
24 97 plates, or differentiated HepaRG cells (24-well plate) or PHHs seeded at 5×10^5 cells/well were used
25
26 98 (see section *cell culture*). The following day, cells were infected with a multiplicity of infection (MOI)
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28 99 of 0.5–2 or 1×10^5 FFU per well (HLC). Erlotinib (33 μM , MedChemExpress, Cat. Nr. HY-50896), EGF
29
30 100 (16.5 nM, 100 ng/mL, MedChemExpress, Cat. Nr. HY-P7109), Cetuximab (34 nM, MedChemExpress,
31
32 101 Cat. Nr. HY-P9905) and Ribavirin (50 μM , Sigma-Aldrich, Cat. Nr. R9644) were applied
33
34 102 simultaneously to virus inoculum. A medium change of infected PHHs was performed 16 h post
35
36 103 infection (p.i.) to fresh William's medium E with supplements for PHHs, Rbv was reapplied. The
37
38 104 medium was changed of infected HLCs at 24 h p.i.. PHHs were fixed 3 d p.i. with 3% PFA and HLCs
39
40 105 7 d p.i. while other assays were fixed 5 d p.i. for immunofluorescence staining of ORF2 protein and
41
42 106 determination of FFUs/well or % ORF2 protein positive cells/image section.

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46 47 108 *HEV Infection Assays with Enveloped HEVcc*

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49 109 For infection assays using enveloped HEVcc, either 1×10^4 HepG2/C3A cells were seeded on collagen-
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51 110 coated 96-well plates one day prior to infection with 200 μL /well of enveloped HEVcc (MOI 0.02–
52
53 111 0.05). The inoculum was removed 24 h p.i. and fresh MEM complete supplied. The indicated
54
55 112 modulators were applied in the inoculum at the time of infection as well as renewed and applied into
56
57 113 the fresh MEM complete after the medium change. Cells were fixed 5 d p.i. or immunofluorescence
58
59 114 staining of ORF2 protein and determination of FFUs/well.

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3 115 *HEV Attachment Assay*
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5 116 For attachment assays, 1×10^4 HepG2/C3A cells were seeded on collagen-coated 96-well plates. The
6
7 117 following day, the cells were pretreated with EGFR modulators for 30 min at 37 °C and the plate then
8
9 118 put on ice for 30 min before addition of ice-cold non-enveloped HEVcc(p6) (MOI 1–2) and the tested
10
11 119 modulator and incubation for another 2 h on ice. Afterwards, the medium was removed and the cells
12
13 120 washed thrice with ice-cold PBS before either the RNA was isolated for qPCR analysis using the RNasy
14
15 121 Mini Kit (Qiagen, Cat. Nr. 74104) following manufacturer's instructions or the cells were supplied with
16
17 122 fresh MEM complete and incubated for 5 days at 37 °C in a 5% (v/v) CO₂ incubator. The cells were
18
19 123 then fixed with 3% PFA and subjected to an immunofluorescence staining of ORF2 protein and
20
21 124 determination of FFUs/well as described below.
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26 126 *HEV Postbinding Assay*
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28 127 For postbinding assays, 1×10^4 HepG2/C3A cells were seeded on collagen-coated 96-well plates. The
29
30 128 following day, the plate was incubated on ice for 30 min prior to infection with non-enveloped HEVcc
31
32 129 p6 (MOI 0.5–2) on ice. The inoculum was left on the cells on ice for 2 h before removal. The cells were
33
34 130 washed thrice with ice-cold PBS and fresh medium supplied with the respective modulator was added
35
36 131 and the cells incubated at 37 °C in a 5% (v/v) CO₂ incubator for either 8 h or 3 days. The medium was
37
38 132 changed at the indicated time and the cells washed with PBS thrice and fresh medium without
39
40 133 modulators was added. The cells were then incubated at 37 °C in a 5% (v/v) CO₂ incubator until fixation
41
42 134 3 d p.i. with 3% PFA and subjected to an immunofluorescence staining of ORF2 protein and
43
44 135 determination of FFUs/well as described below.
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49 137 *HEV Entry Assay*
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51 138 For entry assays, 3.5×10^3 HepG2(-derived) cells were seeded on collagen-coated 96-well plates. The
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53 139 following day, cells were pretreated for 30 min with the respective modulator before infection with non-
54
55 140 enveloped HEVcc (MOI 0.5–2) for 6–8 h. Medium was removed and fresh DMEM complete with
56
57 141 neither virus nor modulator was added except for ribavirin (Rbv), which was added into the fresh
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3 142 medium again. Cells were incubated for 5 d p.i. before fixation with 3% PFA, immunofluorescence
4
5 143 staining of ORF2 protein and determination of FFUs/well as described below.
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9 145 *HEV Luciferase Replication Assays*

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11 146 HEV replication was monitored using a *Gaussia* luciferase (Gluc) construct replacing the ORF2 in the
12
13 147 HEV Kernow-C1-p6 genome⁵. In order to measure the luciferase activity, the respective cells were
14
15 148 electroporated with HEV Kernow-p6-Gluc IVTs. 20 μ L of the supernatant were collected at the
16
17 149 indicated hours post electroporation (h p.e.) and transferred to a white, flat-bottom microplate (Greiner
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19 150 Bio-One, Cat. Nr. 655074). The supernatant was subsequently incubated with luciferase substrate
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21 151 (1 μ mol/L of coelenterazin in PBS, Carl Roth, Cat. Nr. 4094.3) and luciferase activity was measured in
22
23 152 a luminometer (CentroXS3 LB960, Berthold technologies).
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28 154 *RT-qPCR*

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30 155 HEV RNA was quantified using a TaqMan® probe (5'-6FAM-TGATTCTCAGCCCTTCGC-BBQ-3')
31
32 156 one step RT-qPCR based on the GoTaq® Probe 1-Step RT-qPCR System (Promega). 50 ng of isolated
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34 157 RNA were used as template and 5'-GGTGGTTTCTGGGGTGAC-3' (sense) and 5'-
35
36 158 AGGGGTTGGTTGGATGAA-3' (antisense) as HEV primers were utilized⁶. An RNA transcript served
37
38 159 as a standard to quantify RNA copy numbers by serial dilution⁴. All RT-qPCR were run on a
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40 160 LightCycler 480 system (Roche).
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45 162 *Immunofluorescence Staining and Microscopy*

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47 163 Cells were fixed by applying 3% PFA for at least 10 min followed by permeabilization in 0.1% Triton
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49 164 X-100 (Carl Roth, Cat. Nr. 3051.3) in 1 \times PBS for 5 min. Subsequently, the cells were blocked in 5%
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51 165 horse serum (Gibco, Cat. Nr. 26050-088) in 1 \times PBS for a minimum of 1 h. EGFR expression was
52
53 166 stained with a polyclonal goat antibody (R and D Systems, Cat. Nr. AF231, 1:500 in 5% horse-serum),
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55 167 EGFR phosphorylation at Tyr1068 was visualized with monoclonal rabbit antibody (Cell Signaling
56
57 168 Technology, Cat. Nr. 3777S, 1:200 in 5 % horse-serum), for staining of albumin polyclonal anti-rabbit
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59 169 antibody (Agilent, Cat. Nr. A0001, 1:500 in 5% horse-serum) was used, and for the capsid protein
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3 170 (ORF2 protein) a polyclonal HEV genotype 3 capsid protein-specific rabbit hyperimmune serum
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5 171 (diluted 1:5 000 in 5% horse serum, kindly gifted by Prof. Rainer G. Ulrich, Friedrich Loeffler Institute,
6
7 172 Germany ⁷) was used and cells with the respective antibody incubated at 4 °C on a rocking shaker
8
9 173 overnight. Unbound primary antibody was removed by washing twice with 1× PBS and the secondary
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11 174 antibody (goat anti-rabbit AlexaFluor 488 or rabbit-anti-goat AlexaFluor 488, 1:1 000 in 5% horse
12
13 175 serum, Invitrogen, Cat Nr. A-11008 and A-11078, respectively) was added. After 2 h in the dark on a
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15 176 rocking shaker, the cells were washed twice with 1× PBS and the DNA labelled with either 4',6'-
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17 177 diamidino-2-phenylindole (Invitrogen, Cat. Nr. D1306, DAPI, 1:10,000 in H₂O) for 5 min or
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19 178 Hoechst 33342 (Thermo Fisher Scientific, 1:1000 in H₂O). Afterwards, the cells were washed twice
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21 179 with water and stored in water at 4 °C until imaging. All staining steps were performed at room
22
23 180 temperature unless otherwise stated. Images of fluorescently stained cells were taken with a Keyence
24
25 181 BZX800 microscope with 4×, 10× or 20× objectives or with the Zeiss Cell discoverer 7 (CD7)
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27 182 microscope.
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32 184 *Western Blots*

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34 185 For western blot analysis, cells were lysed in either M-Per buffer (Thermo Scientific, Cat. Nr. 78501)
35
36 186 supplemented with Pierce Protease Inhibitor Mix (Thermo Scientific, Cat. Nr. A32953) or RIPA buffer
37
38 187 containing the cOmplete Mini Protease Inhibitor Cocktail (Roche). Cells were centrifuged at 10 000×g
39
40 188 for 15 min, the supernatant then heated at 95°C for 5 min with 1× Laemmli-Buffer and resolved by
41
42 189 SDS-PAGE before being transferred to either nitrocellulose or polyvinylidene fluoride (PVDF)
43
44 190 membranes by wet tank electroblotting. Membranes were blocked with 5% milk in PBS containing
45
46 191 0.05% Tween (PBS-T) for min. 1 h at room temperature and subsequently incubated with primary rabbit
47
48 192 monoclonal anti-EGFR antibody (Cell Signaling Technologies, Cat. Nr. 4267S, 1:1000 in 0.5% milk),
49
50 193 overnight at 4 °C, followed by incubation with secondary horseradish peroxidase (HRP) conjugated
51
52 194 polyclonal goat anti-rabbit antibody (Abcam, Cat. Nr. #ab97051, 1:10 000 or Jackson Immuno Reseach,
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54 195 1:4 000, in 0.5% milk) for 2 h at room temperature. The primary antibody targeting β-actin (Sigma-
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56 196 Aldrich, Cat. Nr. A3854, 1:10 000 in 0.5% milk,) was already conjugated with HRP and was thus
57
58 197 incubated for 2 h after blocking. Subsequently, membranes were developed using the Pierce ECL
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3 198 Western Blotting Substrate (Thermo Scientific, 32109) and analyzed using a chemiluminescence
4
5 199 imaging machine (Celvin S 420, Biostep Sarstedt, or INTASELL Chemostar imager).
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9 201 *Cell Viability Assay*

11 202 To determine the cell viability upon treatment with the different drugs, an MTT (3-(4,5-dimethylthiazol-
13 203 2-yl)-2,5-diphenyltetrazolium bromide) assays was performed. For this, 2.5×10^4 HepG2 cells were
15 204 seeded on collagen-coated 96-well plates. The following day, cells were treated with the indicated
17 205 amounts of modulator. Three days post treatment, MTT substrate (Biomol, Cat. Nr. 15655) supplied in
19 206 DMEM complete medium was added to the cells and incubated at 37 °C in a 5% (v/v) CO₂ incubator
21 207 for 2 hours or until cells have metabolized the substrate to MTT formazan. The medium was removed,
23 208 50 µL DMSO added to each well and the absorbance photometrically measured at 570 nm using the
25 209 Tecan Sunrise Remote plate reader. Cells treated with 70% ethanol served as background control.
27

28 210

30 211 *scRNA Seq*

32 212 Single-cell RNA Sequencing data was mined from Aizarani et al. ⁸. RNA expression was visualized by
34 213 T-distributed stochastic neighbor-embedding (t-SNE) plots via the human liver cell atlas webservice
36 214 (<http://human-liver-cell-atlas.ie-freiburg.mpg.de/>). Violin plots were computed with in-house R script
38 215 using the following packages: SingleCellExperiment, Tidyverse, Scater, Scan.
40 216

42 217 *Statistical Analysis and Software*

44 218 Statistical analysis was performed using GraphPad Prism v9.12 for Windows (La Jolla, CA, USA,
46 219 www.graphpad.com). Dose-dependent treatment was plotted and adjusted to a non-linear regression
48 220 mode using GraphPad Prism. To test significance of mean differences, either student t-test or one-way
50 221 ANOVA followed by Dunnett's multiple comparison test were used, p values < 0.05 (*), <0.01 (**),
52 222 <0.001 (***) and <0.0001 (****) were considered statistically significant. p values >0.05 were
54 223 considered to be non-significant (ns). For image analysis Fiji-ImageJ (v1.53q) ⁹ and CellProfiler (v.
56 224 4.0.7) (www.cellprofiler.org) were used. Graphics were prepared using GraphPad Prism v9.12 for

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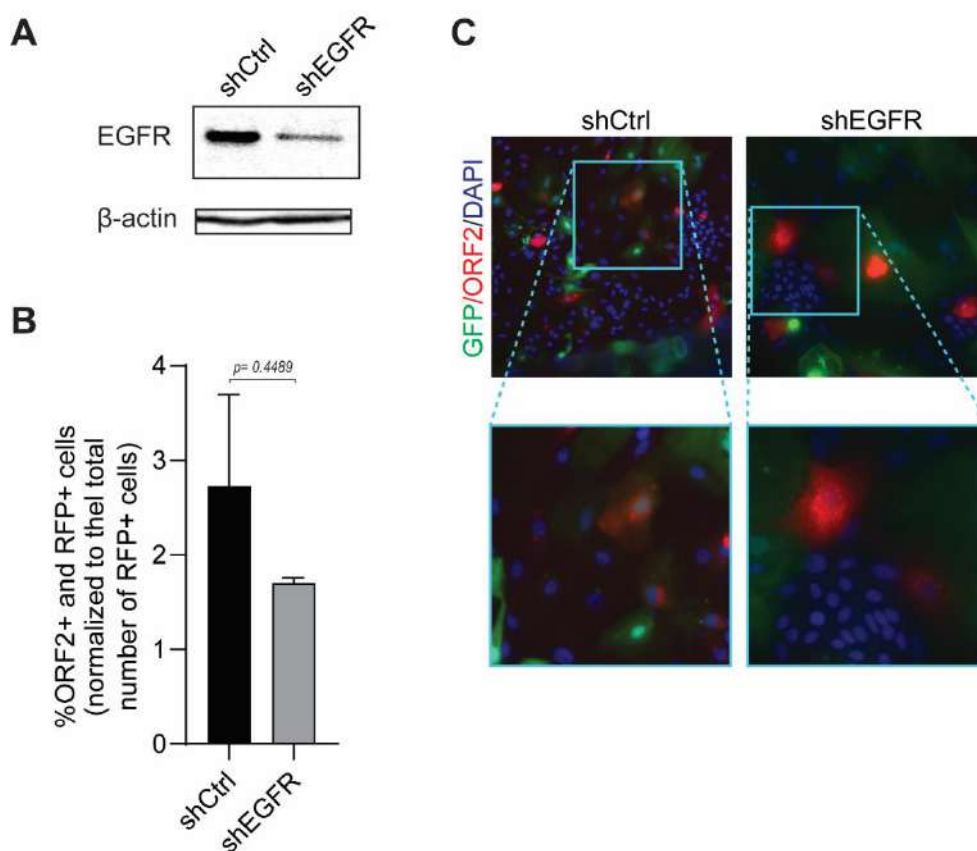
225 Windows (La Jolla, CA, USA, www.graphpad.com), Adobe Illustrator v26.0.3 (www.adobe.com) and
226 BioRender (www.biorender.com).

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For Peer Review

228 **Supplementary Tables and Figures**

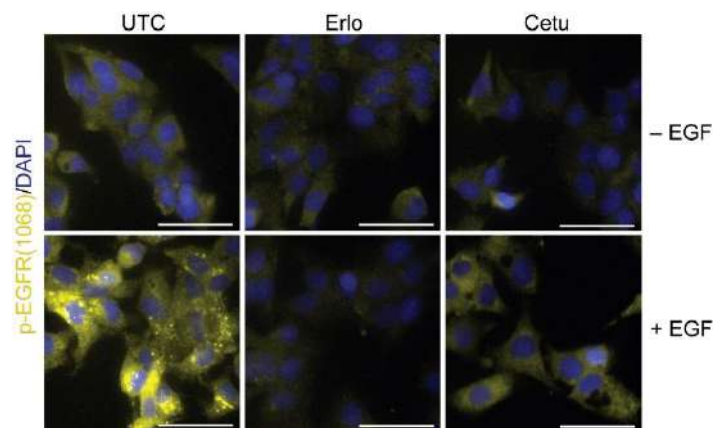
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231 **Supplementary Figure S1: Knock down of EGFR in HLCs reduces HEV infection.** (A) EGFR
 232 protein expression in iPSC-derived HLCs 48 h post transduction with AAVs carrying shRNA targeting
 233 EGFR (shEGFR) or control RNA (shCtrl). (B) Quantification of HEV-infected and shRNA-transduced
 234 (ORF2+, RFP+) HLCs normalized to the total number of HLCs transduced (RFP+).
 235 (ORF2+, RFP+) / ((ORF2+, RFP+) + (ORF2-, RFP+)). (C) Representative immunofluorescence
 236 images stained for ORF2 protein (red). Transduced cells carrying a GFP reporter are indicated in green.
 237 Infection experiments were performed in duplicates with at least 20 frames counted. Mean and SEM
 238 are depicted from at least two independent experiments.

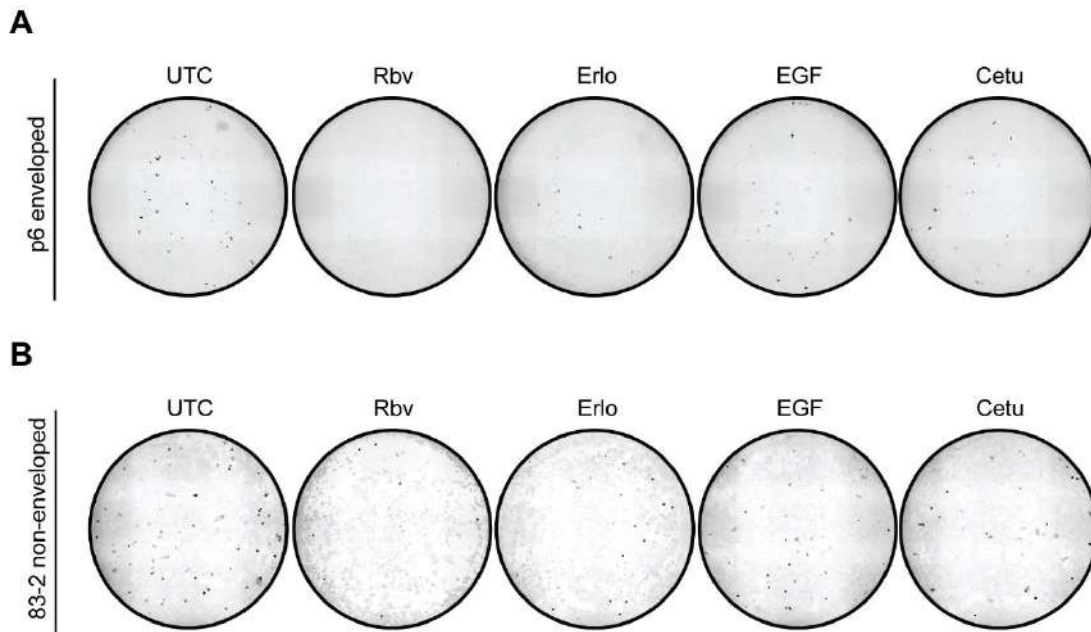
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241 **Supplementary Figure S2: Erlotinib and Cetuximab inhibit EGF induced phosphorylation of**
242 **EGFR at Tyr1068.** Immunofluorescence analysis of EGFR phosphorylation at Tyr1068 after overnight
243 FCS starvation of EGFR-WT ectopically expressing HepG2 cells and after treatment with Erlotinib
244 (33 μ M, Erlo), or Cetuximab (34 nM, Cetu) for 30 min prior to challenge with EGF (16.5 nM) for
245 15 min. Scalebars = 100 μ m.

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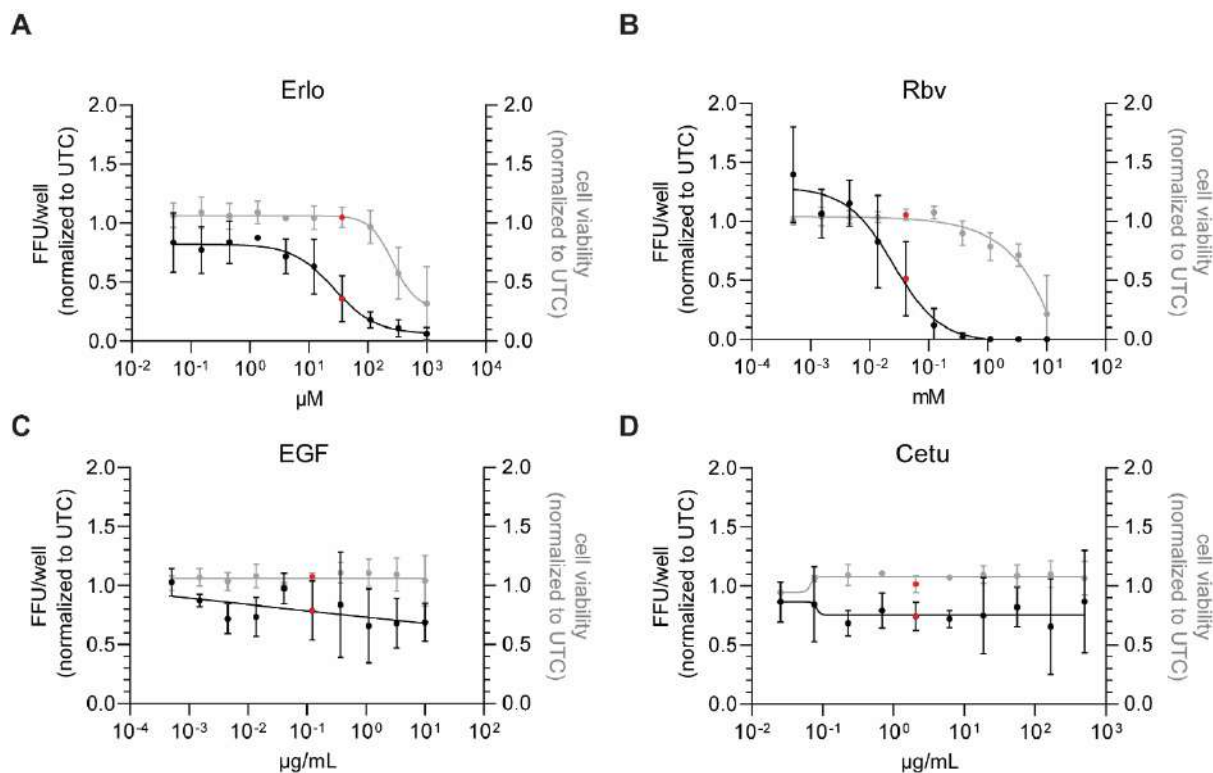


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248 **Supplementary Figure S3: Endogenous EGFR is critical for HEV infection.** (A) HEVcc p6
249 enveloped and (B) 83-2 non-enveloped infection in HepG2 cells under treatment of EGFR modulators
250 Erlotinib (33 μ M, Erlo), EGF (16.5 nM) and Cetuximab (34 nM, Cetu) compared to untreated control
251 cells (UTC), while the HEV inhibitor Ribavirin (50 μ M, Rbv) served as control. HEVcc infected cells
252 were stained against ORF2 protein (indicated in black) and images taken using the 4x magnification of
253 the Keyence microscope. Images were stitched and processed using Fiji. Full well images are depicted.

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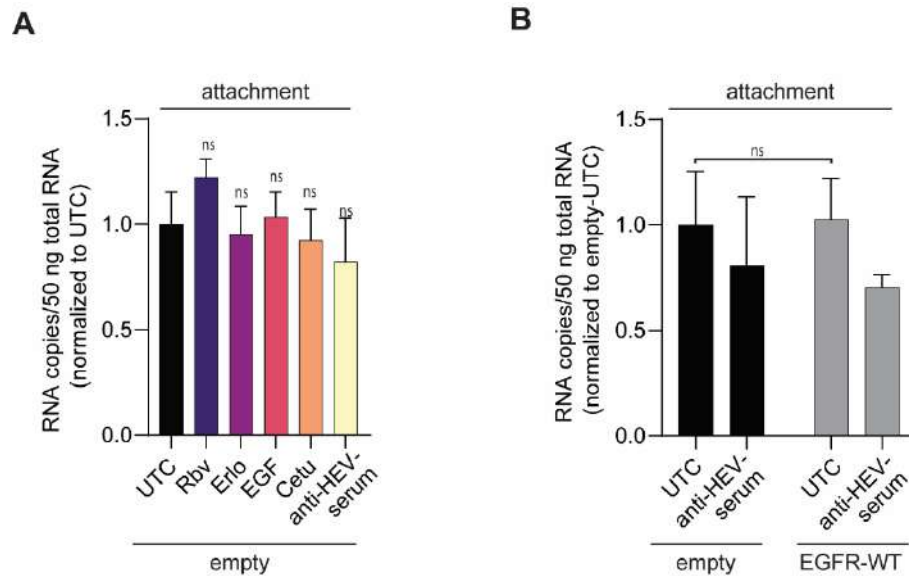


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257 **Supplementary Figure S4: EGFR kinase inhibitor Erlotinib inhibits HEVcc p6 infection in a dose-**
 258 **dependent manner.** Infection with HEVcc p6 under simultaneous treatment of **(A)** Erlotinib (Erlo)
 259 **(B)** Ribavirin (Rbv) **(C)** EGF and **(D)** Cetuximab (Cetu) with the indicated concentrations fixed at
 260 3 d p.i.. FFU/wells (black) normalized to untreated cells. Cell viability (grey) measured using an MTT
 261 Assays at 3 days post treatment and normalized to untreated cells. Indicated in red is the concentration
 262 used in subsequent infection assays with the specific modulator. All experiments were performed in
 263 triplicates. Mean and SEM are depicted from three independent experiments. Dose-dependent treatment
 264 was plotted and adjusted to a non-linear regression model.

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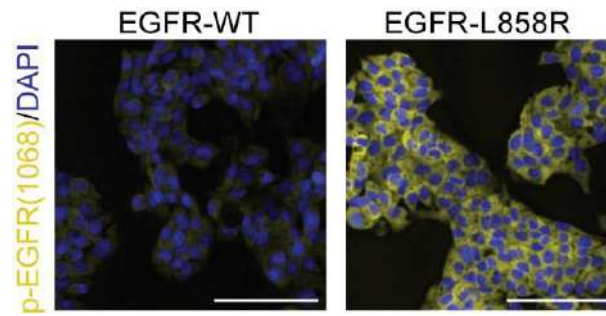


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268 **Supplementary Figure S5: EGFR does not affect HEV attachment analyzed via qPCR.**

269 Quantification of HEVcc (p6) attachment under EGFR modulator treatment RNA copies/50 ng total
 270 RNA isolated and normalized to untreated, infected HepG2/empty cells. (A) HepG2-empty cells were
 271 pretreated with EGFR modulators for 30 min at 37 °C before addition of virus for 2 h on ice, allowing
 272 attachment but not entry. HEV inhibitor Rbv served as negative control here and anti-HEV serum
 273 (1:200) as positive control neutralizing HEVcc (p6). Cells were washed thrice before RNA isolation
 274 and quantification of HEV RNA copies. (B) Either HepG2-empty cells or EGFR-WT ectopically
 275 expressing HepG2 cells were pretreated with anti-HEV serum for 30 min at 37 °C before addition of
 276 virus for 2 h on ice. Cells were washed thrice before RNA isolation and quantification of HEV RNA
 277 copies. To test significance of mean differences, student t-test (B) and one-way ANOVA followed by
 278 Dunnett's multiple comparison test (A) were used, p values < 0.05 (*), <0.01 (**), <0.001 (***) and
 279 <0.0001 (****), p values >0.05 were considered to be non-significant (ns). All experiments were
 280 performed in duplicates. Mean and SEM are depicted from three independent experiments.

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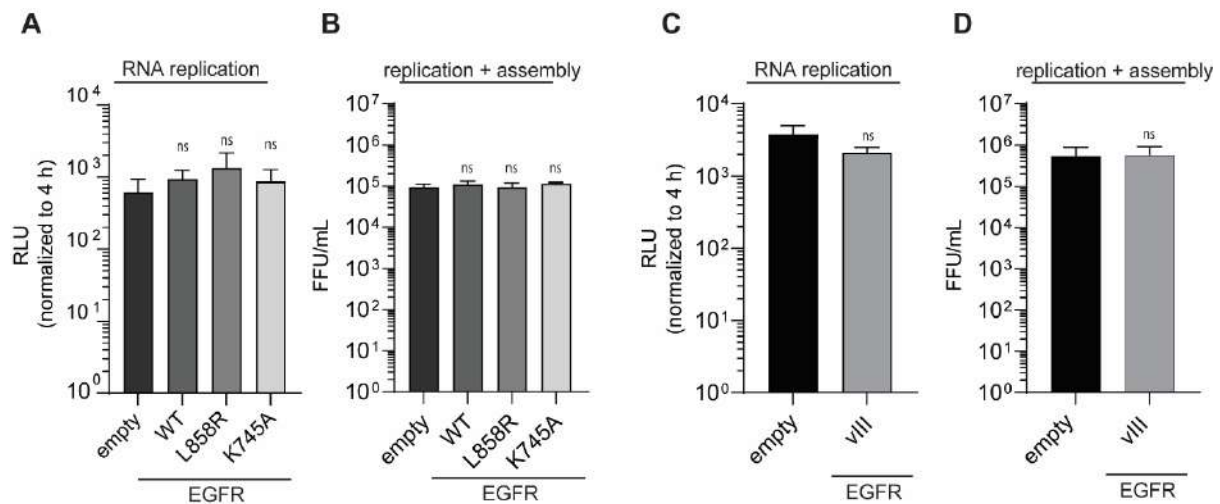
283 **Supplementary Figure S6: EGFR-L858R Mutant is constitutively active.** Immunofluorescence

284 analysis of EGFR phosphorylation at Tyr1068 after overnight FCS starvation of EGFR-WT and

285 EGFR.L858R ectopically expressing HepG2 cells. Scalebars = 100 μ m.

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289 **Supplementary Figure S7: Mutant EGFR expression does not affect HEV RNA replication nor**
 290 **production of progeny virus. (A, C) HEV (p6) replication level in RNA subgenomic replicon (SGR)**
 291 **system 72 h.p.e in mutant EGFR ectopically expressing HepG2 cells normalized to relative light unit**
 292 **(RLU) levels at 4 h.p.e.. (B, D) Mutant EGFR ectopically expressing HepG2 cells transfected with HEV**
 293 **Kernow-p6 RNA for virus production. Virus titers determined from non-enveloped virus produced in**
 294 **mutant EGFR ectopically expressing HepG2 cells. To test significance of mean differences, student t-**
 295 **test (C and D) and one-way ANOVA followed by Dunnett's multiple comparison test (A and B) were**
 296 **used, p values < 0.05 (*), < 0.01 (**), < 0.001 (***) and < 0.0001 (****), p values > 0.05 were considered**
 297 **to be non-significant (ns). Replication experiments were performed in triplicates (A and C),**
 298 **determination of viral titers was performed in duplicates (B and D). Mean and SEM are depicted from**
 299 **three independent experiments.**

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