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Identification of class II ADP-ribosylation factors as cellular factors required for hepatitis C virus replication

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6 3 **Identification of class II ADP-ribosylation factors as cellular**
7 **factors required for hepatitis C virus replication**
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14 7 **Running title: Class II Arfs and HCV replication**
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3 22 **SUMMARY**
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5 23 GBF1 is a host factor required for hepatitis C virus (HCV) replication. GBF1 functions as
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7 24 a guanine nucleotide exchange factor (GEF) for G-proteins of the Arf family, which
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9 25 regulate membrane dynamics in the early secretory pathway and the metabolism of
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11 26 cytoplasmic lipid droplets. Here we established that the Arf-GEF activity of GBF1 is
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13 27 critical for its function in HCV replication, indicating that it promotes viral replication by
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15 28 activating one or more Arf family members. Arf involvement was confirmed with the use
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17 29 of two dominant-negative Arf1 mutants. However siRNA-mediated depletion of Arf1,
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19 30 Arf3 (class I Arfs), Arf4 or Arf5 (class II Arfs), which potentially interact with GBF1, did
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21 31 not significantly inhibit HCV infection. In contrast, the simultaneous depletion of both
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23 32 Arf4 and Arf5, but not of any other Arf pair, imposed a significant inhibition of HCV
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25 33 infection. Interestingly, the simultaneous depletion of both Arf4 and Arf5 had no impact
26
27 34 on the activity of the secretory pathway and induced a compaction of the Golgi and an
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29 35 accumulation of lipid droplets. A similar phenotype of lipid droplet accumulation was
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31 36 also observed when GBF1 was inhibited by brefeldin A. In contrast, the simultaneous
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33 37 depletion of both Arf1 and Arf4 resulted in secretion inhibition and Golgi scattering, two
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35 38 actions reminiscent of GBF1 inhibition. We conclude that GBF1 could regulate different
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37 39 metabolic pathways through the activation of different pairs of Arf proteins.
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41 INTRODUCTION

42 Hepatitis C virus (HCV) is a positive-sense single-stranded RNA virus of the *Flaviviridae*
43 family. Like other positive RNA viruses, HCV genome is replicated in the cytoplasm of its
44 host cell. During HCV replication, internal membranes of the cell are rearranged and
45 these rearranged membranes likely are replication sites of the viral RNA genome. HCV-
46 induced membrane rearrangements have been named membranous web (Egger *et al.*,
47 2002). They include double membrane vesicles (DMV) and clusters of small single
48 membrane vesicles of ER origin (Ferraris *et al.*, 2010; Romero-Brey *et al.*, 2012). DMV
49 have also been observed in cells replicating RNA viruses of other families, including
50 *Picornaviridae* (Limpens *et al.*, 2011; Belov *et al.*, 2012) and *Coronaviridae* (Knoops *et al.*,
51 2008; Ulasli *et al.*, 2010), but not with other viruses of the *Flaviviridae* family (Welsch *et*
52 *al.*, 2009; Gillespie *et al.*, 2010; Schmeiser *et al.*, 2014).

53 GBF1, a major regulator of membrane dynamics in the early secretory pathway, has
54 recently emerged as a host factor involved in the replication of several viruses of the
55 *Picornaviridae* (Belov *et al.*, 2008; Lanke *et al.*, 2009), *Coronaviridae* (Verheije *et al.*,
56 2008), and *Flaviviridae* (Goueslain *et al.*, 2010; Carpp *et al.*, 2014) families. GBF1 is a
57 brefeldin A (BFA)-sensitive guanine nucleotide exchange factor (GEF) for G-proteins of
58 the Arf family (Claude *et al.*, 1999). Arfs recruit and activate a number of effectors, which
59 function in vesicular transport, phospholipid metabolism, actin cytoskeleton regulation
60 and lipid droplet metabolism (Donaldson and Jackson, 2011; Wright *et al.*, 2014). There
61 are 6 Arfs and 16 Arf-like proteins in mammalian cells. Arfs are divided into three
62 classes based on sequence homology. Class I contains Arf1-3, class II contains Arf4 and
63 Arf5, and Arf6 constitutes the sole member of class III (Donaldson and Jackson, 2011;
64 Wright *et al.*, 2014). As an ArfGEF, GBF1 shows selectivity for Arfs of classes I and II
65 (Claude *et al.*, 1999; Szul *et al.*, 2007). GBF1 is a large protein of 206 kDa containing 6

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3 66 conserved domains (Bui *et al.*, 2009). The GEF activity is catalyzed by the Sec7 domain.
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5 67 Our knowledge of the functions of the other conserved domains of GBF1 is still scarce
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7 68 (Bui *et al.*, 2009; Belov *et al.*, 2010; Bouvet *et al.*, 2013).
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10 69 The mechanism of action of GBF1 in viral infection is not yet understood. Several lines of
11
12 70 evidence indicate that GBF1 is required (Verheije *et al.*, 2008; Goueslain *et al.*, 2010;
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14 71 Carpp *et al.*, 2014) and/or is recruited to viral replication complexes (Verheije *et al.*,
15
16 72 2008; Richards *et al.*, 2014) at the onset of viral replication, but not at later time points.
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19 73 It is generally assumed to function as an ArfGEF activating Arf1, which in turn would
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21 74 recruit the COP-I coatomer, a molecular machinery involved in intracellular transport,
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23 75 which has also been reported to be required for the replication of several positive strand
24
25 76 RNA viruses, including HCV (Gazina *et al.*, 2002; Cherry *et al.*, 2006; Tai *et al.*, 2009;
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27 77 Wang *et al.*, 2012). Accordingly, a GBF1-Arf1-COP-I pathway has been proposed to play a
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29 78 role in the replication of HCV (Matto *et al.*, 2011; Zhang *et al.*, 2012; Farhat *et al.*, 2013)
30
31 79 and other viruses (Wang *et al.*, 2014). However, a direct functional link between GBF1
32
33 80 activation and COP-I function in HCV replication has not been experimentally
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35 81 demonstrated. For instance, in poliovirus infection, the transient recruitment of GBF1 at
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37 82 replication complexes is not coupled to COP-I recruitment (Richards *et al.*, 2014),
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39 83 suggesting the existence of distinct functions for GBF1 at viral replication complexes and
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41 84 in the secretory pathway of the cell. Indeed, GBF1 may primarily function in poliovirus
42
43 85 infection by activating or recruiting other cellular effectors essential for viral replication
44
45 86 in an Arf1-independent manner (Belov *et al.*, 2010). During HCV replication, the
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47 87 activation by GBF1 of an Arf1 effector different from COP-I, the phosphatidylinositol 4-
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49 88 kinase III β , has been proposed to be involved in HCV replication (Zhang *et al.*, 2012).
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51 89 However the involvement of this kinase during HCV replication is still controversial (Tai
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53 90 *et al.*, 2009; Vaillancourt *et al.*, 2009; Berger *et al.*, 2009; Borawski *et al.*, 2009; Trotard *et*
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3 91 *al.*, 2009). Other possibilities for GBF1 function during viral replication include the
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5 92 activation of other members of the Arf family or mechanisms unrelated to Arf activation.
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7 93 For example, the mechanism of action of GBF1 during poliovirus replication has been
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10 94 demonstrated not to depend on its catalytic Sec7 domain and therefore on its GEF
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12 95 activity (Belov *et al.*, 2010). In this study, we investigated the mechanism of action of
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14 96 GBF1 in HCV replication. Our data support a model in which class II Arf proteins mediate
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16 97 GBF1 function.
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20 21 99 **RESULTS**

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23 100 ***Role of the Sec7 domain of GBF1 in HCV infection.*** It has recently been reported that
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25 101 GBF1 but not Arf activation is required for poliovirus replication (Belov *et al.*, 2010),
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27 102 suggesting that GBF1 may have other functions than Arf activation during a viral
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29 103 infection. More specifically, a catalytically inactive GBF1 truncation mutant lacking the
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31 104 Sec7 domain has been reported to be sufficient for rescuing poliovirus replication from
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33 105 BFA inhibition. To investigate if GBF1 could also function in a Sec7-independent manner
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35 106 in HCV replication, we expressed a series of GBF1 truncation mutants (figure 1A) in
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37 107 Huh-7 cells and infected them with HCV in the presence of BFA. We used a dose of 50
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39 108 ng/ml BFA, which decreases HCV infection about 10 times and has minimal cytotoxic
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41 109 effects in Huh-7 cells during the time scale of the HCV infection assay (Goueslain *et al.*,
42
43 110 2010). We previously showed that BFA has no impact on HCV entry and inhibits the
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45 111 replication step of the HCV life cycle (Goueslain *et al.*, 2010). GBF1 construct expression
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47 112 and HCV infection were probed by immunoblotting at 30 hpi. At this time, only cells
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49 113 initially infected are positive (Afzal *et al.*, 2014), implying that the impact of BFA
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51 114 inhibition of progeny virion release on HCV protein expression is negligible.
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3 115 BFA treatment had no impact on the expression of GBF1 constructs (figure 1B). In the
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5 116 absence of BFA, similar E1 expression levels were observed with all GBF1 constructs,
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7 117 indicating that none of these constructs had any dominant-negative effect on HCV
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9 118 infection. In the presence of BFA, E1 expression levels were reduced about 10-20 times
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11 119 in untransfected cells or in cells expressing YFP. HCV infection was partially rescued in
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13 120 cells expressing full-length wild-type or M832L BFA-resistant constructs, whereas the
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15 121 E794K inactive mutant was unable to rescue HCV infection in the presence of BFA, as
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17 122 previously reported (Goueslain *et al.*, 2010). Importantly, unlike for poliovirus, all the C-
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19 123 terminal deletion constructs including GBF1(1-710), which lacks a Sec7 domain, were
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21 124 unable to rescue HCV infection in these complementation experiments (figure 1B and C).
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23 125 Therefore, these results support a Sec7-dependent function of GBF1 in HCV infection.
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25 126 As a control, we also quantified the impact of GBF1 constructs on the secretion of human
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27 127 serum albumin (HSA) by BFA-treated Huh-7 cells. A well-established function of GBF1 is
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29 128 the control of membrane dynamics in the early secretory pathway, and this function
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31 129 relies on Arf activation. Therefore, HSA secretion is a marker of GBF1-mediated Arf
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33 130 activation in these experiments. The treatment of Huh-7 cells with 50 ng/ml BFA
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35 131 decreased albumin secretion in control mock-, or YFP-transfected cells (figure 1D). As
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37 132 expected, wild type and M832L GBF1 constructs partially restored albumin secretion,
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39 133 but none of the other constructs did. These results indicate a similar requirement of
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41 134 GBF1 domains for HCV infection and for Arf activation and are consistent with a
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43 135 requirement of ArfGEF activity to promote HCV infection.
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53 137 ***Arf1 dominant negative mutants inhibit HCV infection.*** Our results with GBF1
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55 138 truncation mutants suggested that the activation of at least one BFA-sensitive Arf family
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57 139 member is required for HCV infection. We further investigated this question using two
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3 140 different mutants of Arf1 affecting either GDP exchange (T31N) or GTP hydrolysis
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5 141 (Q71L). When overexpressed, these two constructs act as dominant negative mutants in
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7 142 a protein transport assay and block the intracellular traffic at different steps of the early
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9 143 secretory pathway (Dascher and Balch, 1994). Arf mutants were expressed as EGFP or
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11 144 mCherry fusion proteins in Huh-7 cells and the cells were infected with HCV. The
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13 145 infection was monitored by immunofluorescence (figure 2A) and the percentage of
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15 146 fluorescent cells infected was quantified. As expected, the expression of the T31N
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17 147 mutant, which has a BFA-like effect, strongly inhibited HCV infection (figure 2B). The
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19 148 Q71L mutant, which is locked in the active, GTP-bound form, also strongly inhibited HCV
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21 149 infection, and the wild type Arf1 construct had a moderate impact compared to EGFP
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23 150 and mCherry controls. On the other hand, Arf1 constructs did not have any effect on
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25 151 adenovirus infection, indicating that the effects observed with HCV did not result from
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27 152 any cytotoxic effects of Arf1 constructs. These results confirmed the involvement of an
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29 153 Arf family member in HCV infection.
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37 155 ***Role of Arf family members in HCV infection.*** The results with dominant negative
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39 156 mutants of Arf1 suggest that Arf1 and/or other Arf family members are involved in HCV
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41 157 infection. We used siRNA technology to determine which Arf proteins are specifically
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43 158 involved in HCV infection. Given the BFA sensitivity of HCV infection, we focused our
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45 159 study on class-I and class-II Arfs. Arf1, Arf3, Arf4 and Arf5 (human cells have no Arf2)
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47 160 were targeted with pools of 4 siRNAs, except for Arf1, which was targeted by 2 siRNAs
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49 161 only, because we found that the commercial Arf1 pool contains 2 individual siRNAs also
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51 162 targeting Arf3 (supplementary figure 1). Surprisingly, the depletion of each Arf protein
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53 163 resulted in a moderate (30-35%) inhibition of HCV infection (figure 3A). To assess the
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55 164 extent of siRNA-mediated Arf depletion, we quantified mRNAs by quantitative RT-PCR.
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3 165 The depletions were specific and reached 87% inhibition for Arf1 and about 95%
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5 166 inhibition for the other Arfs (figure 3B). We also observed an up-regulation of Arf4
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7 167 expression upon Arf1 and to a lesser extent Arf3 depletion, suggesting the existence of
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9 168 compensatory mechanisms regulating the expression of Arf family members.

11 169 Arfs may have overlapping functions, and it has been reported that the depletion of
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13 170 different pairs of Arfs results in specific phenotypes (Volpicelli-Daley *et al.*, 2005).

15 171 Therefore we considered that a specific pair of Arfs could be involved in HCV infection.

17 172 For this reason, we also depleted pairs of Arfs. Again, higher levels of Arf4 mRNA were
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19 173 observed upon Arf1+Arf3 depletion or Arf1+Arf5 depletion, confirming the up-
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21 174 regulation of Arf4 in Arf1-depleted cells (figure 3B). Interestingly, HCV infection was
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23 175 decreased down to 17±8% in cells depleted of both Arf4 and Arf5, whereas the
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25 176 depletion of other pairs did not significantly decrease more HCV infection than single Arf
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27 177 depletions (figure 3A). These results indicate a special importance of class-II Arfs in HCV
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29 178 infection.

31 179 To rule out off-target effects, Arf4 or Arf5 were expressed in siRNA-treated cells. Arf
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33 180 proteins of murine origin were expressed in Huh-7 cells simultaneously depleted of both
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35 181 Arfs. Immunoblot analysis confirmed Arf5 expression (figure 3C). However, Arf4
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37 182 expression could not be confirmed due to the lack of reactivity toward murine Arf4 of
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39 183 the antibody. HCV infection was partially restored in Arf4-expressing cells and in Arf5-
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41 184 expressing cells (figure 3D). We also observed a slight decrease of HCV infection in cells
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43 185 transfected with Arf4 in the control condition (figure 3D). The expression of Arf4 or Arf5
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45 186 in double-depleted cells increased HCV infection up to levels similar to those observed
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47 187 in cells depleted of a single Arf protein (figure 3A). In contrast, no rescue of HCV
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49 188 infection was observed in cells transfected with siRNAs to PI4KA, as expected (figure
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51 189 3D).

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3 190 To determine if the replication step of the HCV life cycle is affected by the simultaneous
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5 191 depletion of class-II Arfs, siRNA-transfected Huh-7 cells were electroporated with an in
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7 192 vitro-transcribed recombinant Δ E1E2 JFH1 genomic RNA expressing a *Renilla* luciferase
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9 193 reporter. Measuring luciferase activity over a 72-h time course assessed replication.
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11 194 Luciferase activity was not inhibited at 4h post electroporation, and was reduced about
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13 195 9 times in cells depleted of Arf4 and Arf5 at 48 and 72 h post electroporation (figure 3E),
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15 196 indicating an inhibition of HCV replication with no impact on the initial translation level
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17 197 of HCV RNA.

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21 198 We also investigated HCV entry in Huh-7 cells simultaneously depleted of both class II
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23 199 Arfs using HCVpp. As controls, we used VSV-Gpp, which rely on endocytosis and pH-
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25 200 dependent fusion for entry, like HCVpp, and RD114pp, which enter by a pH-independent
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27 201 mechanism. All pseudoparticles were similarly inhibited by the double depletion of Arf4
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29 202 and Arf5 (supplementary figure 2), indicating a post-fusion inhibition of pseudoparticle
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31 203 entry. Therefore, we cannot conclude on the function of class II Arfs during the entry
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33 204 step of the HCV life cycle.
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39 206 ***Class II Arfs are not recruited in HCV replication complexes.*** The intracellular
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41 207 localization of Arf4 and Arf5 in Huh-7 cells was analyzed by immunofluorescence
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43 208 confocal microscopy. Cells were transfected with expression vectors for Arf4-GFP or
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45 209 Arf5-GFP. Both Arf4-GFP and Arf5-GFP were observed in Golgi-like perinuclear
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47 210 structures together with a diffuse staining of the cytosol and the nucleus
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49 211 (supplementary figure 3), which is consistent with the dual localization of Arf proteins
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51 212 as membrane-associated and soluble proteins. Arf4- and Arf5-positive perinuclear
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53 213 structures were also labeled with an antibody to GM130 (supplementary figure 3A),
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55 214 indicating that they are localized in the cis-Golgi. Interestingly, Arf4 and Arf5
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57 215 perinuclear structures also colocalized with GBF1 (supplementary figure 3B).
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3 216 In infected cells, the localization of Arf4-GFP and Arf5-GFP was compared to that of NS3
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5 217 and NS5A, two markers of HCV replication complexes. We did not observe any major
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7 218 change of Arf4 and Arf5 patterns (supplementary figure 4), except in Arf4-GFP
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9 219 expressing cells, where the Golgi appears less structured. This tendency toward a less
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11 220 compact Golgi was also observed in non-infected cells (supplementary figure 3). Very
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13 221 few levels of colocalization of Arfs and NS3 (supplementary figure 4A) or NS5A
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15 222 (supplementary figure 4B) were observed, indicating that class II Arfs are not
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17 223 permanently recruited to HCV replication complexes.
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23 225 ***Impact of Arfs depletion on the secretory pathway.*** To get insight into a mechanism of
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25 226 action of the pair of class-II Arfs, we analyzed their involvement in the regulation of
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27 227 secretion. Following depletion of different pairs of Arfs, we monitored the secretion of
28
29 228 serum albumin and VLDL-associated apolipoprotein E (apoE), two proteins expressed
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31 229 and constitutively secreted by Huh-7 cells. Albumin and apoE secretion were inhibited
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33 230 in cells with reduced levels of both Arf1 and Arf4, but were not affected by the depletion
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35 231 of any other pair of Arfs, including Arf4 and Arf 5 (figure 4A). These results indicate that
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37 232 the simultaneous depletion of class II Arfs has no functional impact on the secretory
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39 233 pathway.
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43 234 To further examine the impact of their depletion on the secretory pathway, we also
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45 235 investigated the morphology of cellular compartments. We observed a more compact
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47 236 Golgi morphology in cells simultaneously depleted of Arf4 and Arf5 than in control cells
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49 237 using the cis-Golgi marker GM130 (figure 4B). In contrast, the depletion of Arf1 and Arf4
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51 238 resulted in a fragmentation of the cis-Golgi, as previously reported for HeLa cells
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53 239 (Volpicelli-Daley *et al.*, 2005). A similar scattered Golgi phenotype was observed upon
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55 240 GBF1 depletion (figure 4B) or after a BFA treatment (data not shown). We did not
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3 241 observe any morphological change in cells depleted of other pairs of Arfs
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5 242 (supplementary figure 5). A compaction of the Golgi complex was also observed in class
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7 243 II Arfs-depleted cells with other markers. In these cells, GBF1 and ERGIC53, two proteins
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9 244 located both in the cis-Golgi and the ERGIC, and TGN46, a marker of the trans-Golgi
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11 245 network, all showed a more compact localization (figure 4B), indicating that the effect is
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13 246 not restricted to the cis-Golgi. Again, the depletion of Arf1 and Arf4 or of GBF1 resulted
14
15 247 in a scattered pattern of ERGIC53, GBF1 and TGN46 (figure 4B). No other Arf pair
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17 248 produced similar phenotypes of scattered or compacted patterns with any of the
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19 249 markers (data not shown). These results indicate that the pair of class II Arfs does not
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21 250 contribute to the Golgi scattering effect of GBF1 inhibition.
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252 ***Impact of class II Arfs depletion on the lipid droplets.*** In addition to its role as a
253 regulator of the early secretory pathway, GBF1 is also known to regulate lipid droplets
254 (Guo *et al.*, 2008; Beller *et al.*, 2008; Soni *et al.*, 2009), a cellular compartment playing a
255 critical role in the HCV life cycle. Given the lack of effect of class II Arfs on the secretory
256 pathway, we also investigated the morphology of lipid droplets after Arf depletion. Lipid
257 droplets were stained with BODIBY493/503 and the Golgi was also labeled with an
258 antibody to GM130 to verify the effect of Arf4 and Arf5 depletion. In cells simultaneously
259 depleted of Arf4 and Arf5, lipid droplets were larger than in control cells and were
260 packed together at the periphery, often in one extension of the cell, unlike in control
261 cells where smaller lipid droplets were usually scattered throughout the cytoplasm
262 (figure 5A). A similar effect was observed in cells treated for 24 hours with a low dose of
263 BFA (figure 5B), or in cells depleted of GBF1 (data not shown), suggesting that the action
264 of the pair of class II Arfs on lipid droplets is regulated by GBF1. Importantly, the
265 depletion of other Arf pairs did not result in a similar phenotype of accumulation of

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3 266 enlarged lipid droplets. Lipid droplets were very similar to controls for all depletions
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5 267 except for the pair Arf1 and Arf4, and to some extent the pair Arf3 and Arf4, the
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7 268 depletion of which resulted in a reduced number of enlarged lipid droplets
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10 269 (supplementary figure 5). This result suggests that the pair of class II Arfs could
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12 270 participate in GBF1-mediated regulation of lipid metabolism.
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16 272 **DISCUSSION**

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18 273 In this study we investigated the mechanism of action of GBF1 in HCV infection. Our
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20 274 results suggest that GBF1 functions in HCV infection by activating class II Arfs. This is
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22 275 different from what has been reported for in poliovirus, for which its GEF activity and
23
24 276 Arf activation are not required (Belov *et al.*, 2010). A series of GBF1 truncation mutants
25
26 277 inactive for regulating the secretory pathway of the cell, including a construct lacking a
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28 278 Sec7 domain, were shown to complement BFA inhibition of poliovirus replication. Here
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30 279 we showed that unlike poliovirus, the same series of GBF1 mutants do not support HCV
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32 280 replication, clearly indicating a difference of action for GBF1 in poliovirus and HCV
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34 281 replication. This requirement for GEF activity was confirmed with the use of Arf1
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36 282 dominant negative mutants.
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41 283 The function of GBF1 in HCV replication is also different from its function in membrane
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43 284 traffic, as already suggested by our previous study of BFA resistant cell lines (Farhat *et*
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45 285 *al.*, 2013). Indeed our results suggest that GBF1 may act by activating different pairs of
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47 286 Arf proteins for the control of HCV replication and of protein secretion. The pair Arf1-
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49 287 Arf4 mediates GBF1 control of the secretory pathway, as already reported (Volpicelli-
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51 288 Daley *et al.*, 2005), whereas the pair Arf4-Arf5 mediates GBF1 function in HCV
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53 289 replication. Recently, Arf4 and Arf5 have also been reported to be involved in dengue
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55 290 virus infection, although for this virus, the function of this pair of Arfs was suggested to
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3 291 be involved in the assembly step (Kudelko *et al.*, 2012). Interestingly, the depletion of
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5 292 Arf4 was reported to protect cells from BFA toxicity, and this effect also depends on
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7 293 GBF1, Arf1 and Arf5 (Reiling *et al.*, 2013).

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10 294 Based on siRNA experiments, previous studies, including our own, suggested an
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12 295 involvement of Arf1 in HCV replication (Matto *et al.*, 2011; Zhang *et al.*, 2012; Farhat *et*
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14 296 *al.*, 2013) that we did not observe in this study. However, in none of these previous
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17 297 studies were the results of siRNA depletions confirmed by re-expressing Arf1, in order
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19 298 to rule out off-target effects. Off-target effects are always a potential pitfall with siRNA
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21 299 experiments, as exemplified by our finding that the commercial siRNA pool targeting
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23 300 Arf1 also inhibits Arf3 expression. Reducing siRNA concentration may help to reduce
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25 301 off-target effects. Using a more efficient transfection agent allowed us to decrease siRNA
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27 302 concentration from 80 nM double transfections previously used down to 20 nM single
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29 303 transfections in this study, with better depletion results on control proteins (data not
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31 304 shown). With these new experimental conditions, Arf1 depletion resulted in a lower
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33 305 inhibition of HCV replication (from about 50% in our previous study (Farhat *et al.*,
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35 306 2013) down to about 30% inhibition in this study) despite efficient reduction of Arf1
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37 307 mRNA expression. All single Arf depletions similarly yielded low levels of inhibition of
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39 308 HCV infection, in line with the reported lack of phenotype of single Arf depletion
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41 309 (Volpicelli-Daley *et al.*, 2005). In contrast, we found a stronger inhibition of HCV
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43 310 infection when cells were simultaneously depleted of both Arf4 and Arf5. This finding is
44
45 311 not inconsistent with the inhibition of HCV infection by Arf1 dominant negative mutants,
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47 312 because trans dominant phenotypes often result from interactions with regulators (like
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49 313 Arf-GEFs and Arf-GAPs in this case). This suggests that Arf1, Arf4 and Arf5 share
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51 314 common regulators, as also indicated by their common inhibition by BFA.
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3 315 Specific phenotypes were already reported for different pairs of Arfs (Volpicelli-Daley *et*
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5 316 *al.*, 2005). In our study, the depletion of Arf4 and Arf5 displayed a BFA-like effect on
6
7 317 lipid droplets morphology and on HCV infection that the pair Arf1-Arf4 did not. On the
8
9 318 other hand, the depletion of the Arf1-Arf4 pair resulted in a BFA-like inhibition of HSA
10
11 319 and ApoE secretion, whereas the depletion of the Arf4-Arf5 pair did not. Cells depleted
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13 320 of Arf1 and Arf4 also displayed a scattered Golgi very similar to what is observed in
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15 321 BFA-treated cells or in cells depleted of GBF1, whereas cells depleted of Arf4 and Arf5
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17 322 had a more compact Golgi morphology than control cells. This effect is not mimicked by
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19 323 GBF1 inhibition, but is rather reminiscent of cells treated with latrunculin B, an inhibitor
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21 324 of actin polymerization (Valderrama *et al.*, 2001; Dippold *et al.*, 2009). Therefore, the
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23 325 Arf4-Arf5 pair could potentially activate an effector involved in the regulation of the
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25 326 actin cytoskeleton. Alternatively, we can also speculate that this effect could result from
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27 327 an alteration of the metabolism of phosphoinositides, because actin fibers are linked to
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29 328 Golgi membranes by PI4P-interacting protein GOLPH3 (Dippold *et al.*, 2009). Taken
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31 329 together these results suggest that GBF1 fulfills its different cellular functions by
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33 330 activating different pairs of Arfs, which in turn control different aspects of the cellular
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35 331 metabolism.

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37 332 An intriguing question is how each pair of Arfs controls specific pathways. This appears
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39 333 to be a common feature of G-proteins of the Arf family (Panic *et al.*, 2003; Setty *et al.*,
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41 334 2003; Volpicelli-Daley *et al.*, 2005; Cohen *et al.*, 2007). A redundancy of action is the
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43 335 simplest explanation, although it is quite difficult to match this model with the diversity
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45 336 of phenotypes observed with the depletion of different pairs of BFA-sensitive Arfs
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47 337 (Volpicelli-Daley *et al.*, 2005). Another model for Arf pair specificity could be the
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49 338 formation of Arf dimers, at the site of interaction with effectors. However, such a kind of
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51 339 interactions has not yet been reported for any Arf effector to our knowledge. It is also
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3 340 possible that the recruitment or the activation of one Arf would depend on the activation
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5 341 of the other one. Different models compatible with this possibility have been proposed
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7 342 (Cohen *et al.*, 2007; Chun *et al.*, 2008). A cascade of recruitment has been reported for
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9 343 other Arf family members (Panic *et al.*, 2003; Setty *et al.*, 2003; Cohen *et al.*, 2007;
10
11 344 Christis and Munro, 2012). This hypothesis would be more compatible if similar
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13 345 phenotypes were observed in cells depleted of either protein and in double depleted
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15 346 cells. However, if the selectivity of the system were not stringent, then it could be
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17 347 compatible with an inhibition only visible in double depleted cells. It would be
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19 348 interesting to determine if such a mode of action actually occurs for Arf1-Arf4 and Arf4-
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21 349 Arf5 pairs.
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26 350 In conclusion, our results indicate that the role of GBF1 in HCV replication is mediated
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28 351 by its ArfGEF activity and is potentially related to a function of regulation of lipid
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30 352 metabolism, rather than of regulation of the protein secretory pathway. Interestingly,
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32 353 two members of the Arf family, class II Arf4 and Arf5, appear to be of special importance
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34 354 for mediating GBF1 function in HCV replication. Although very little is known about the
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36 355 specific functions of class II Arfs, data from our study and from another group
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38 356 (Takashima *et al.*, 2011) suggest their involvement in the control of lipid metabolism, as
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40 357 evidenced by the abnormal morphology of lipid droplets in cells depleted of both Arf4
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42 358 and Arf5. However, their mechanism of action is unknown. A role for the drosophila Arf1
43
44 359 homologue Arf79F in controlling the localization of enzymes of the triglyceride
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46 360 metabolism to oleic acid-induced lipid droplets was recently described (Wilfling *et al.*,
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48 361 2014). It is not yet clear how this might relate to our results with class II Arfs-depleted
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50 362 cells, because the depletion of Arf1 reduced the size of lipid droplets in drosophila cells,
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52 363 instead of increasing it as we observed for simultaneous depletion of both Arf4 and Arf5
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54 364 in Huh-7 cells. Further studies will be needed to determine the nature of the enzymes or
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3 365 transporters involved in lipid metabolism that are effectors of Arf4 and Arf5. These
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5 366 proteins may constitute host factors critical for the replication of HCV and potentially
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7 367 other RNA viruses.
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11 369 **EXPERIMENTAL PROCEDURES**

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14 370 **Reagents.** Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline
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16 371 (PBS), goat and fetal calf sera (FCS), BODIBY 493/503 and 4',6-diamidino-2-
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18 372 phenylindole (DAPI) were purchased from Life Technologies. Mowiol 4-88 was from
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20 373 Calbiochem. Protease inhibitors mix (Complete) was from Roche. Other chemicals were
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22 374 from Sigma.

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26 375 **Antibodies.** Mouse anti-E1 mAb A4 (Dubuisson *et al.*, 1994) was produced in vitro by
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28 376 using a MiniPerm apparatus (Heraeus) as recommended by the manufacturer. Mouse
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30 377 anti-NS5A mAb 9E10 (Lindenbach *et al.*, 2005) was kindly provided by C. M. Rice (The
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32 378 Rockefeller University). Mouse anti-NS3 mAb 1848 was from Virostat. Rabbit anti-
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34 379 human Arf4 mAb (ab171746) was from Abcam. Mouse anti-Arf5 mAb 1B4 was from
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36 380 Abnova. Sheep anti-TGN46 was from Serotec. Mouse anti-GBF1 and anti-GM130 mAbs
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38 381 were from Transduction Laboratories. Mouse anti- β -tubulin mAb (TUB 2.1) was from
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40 382 Sigma. Mouse anti-HSA (ZMHSA1) was from Invitrogen. Goat anti-HSA (507313) was
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42 383 from Calbiochem. Mouse anti-GFP mAb was from Roche. Alexa 555-conjugated donkey
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44 384 anti-sheep IgG antibody was from Life Technologies. Peroxidase-conjugated goat anti-
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46 385 mouse, and anti-sheep IgG, and cyanine 3-conjugated goat anti-mouse IgG were from
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48 386 Jackson Immunoresearch.

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53 387 **DNA constructs.** GBF1 deletion mutants were described previously (Niu *et al.*, 2005;
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55 388 Belov *et al.*, 2010). WT Arf1, Q71L and T31N mutants were kindly provided by B.
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57 389 Hoflack (Technische Universität Dresden, Germany). Arf coding sequence was excised
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3 390 from pGEM2 plasmids using *Hind*III and *Bam*HI and subcloned in pEGFP-N1 (Clonetechn)
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5 391 between the same sites to generate Arf1-GFP constructs. To generate Arf1-mCherry
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7 392 constructs, the GFP coding sequence was excised with AgeI and NotI and replaced by
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9 393 mCherry coding sequence PCR-amplified using primers
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11 394 GATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAG and
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13 395 AGAGTCGCGGCCGCTCTACTTGTACAGCTCGTCCATG. Arf4-GFP and Arf5-GFP constructs
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15 396 were as previously described (Chun *et al.*, 2008) and were obtained through Addgene.
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17 397 To generate expression vectors for mouse Arf4 and Arf5 used in rescue experiments, the
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19 398 coding sequences were PCR-amplified from mouse 11-day embryo Marathon-Ready
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21 399 cDNA (Clonetechn) using primers CTTAAGCTTCCGCCATGGGCCTCACCATC and
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23 400 GTAGGATCCTTAACGTTTTGAAAGTTCATTTGAC (Arf4) or
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25 401 CTTAAGCTTCCGCCATGGGCCTCACGGTG and GTAGGATCCCTAGCGCTTTGACAGCTCGT
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27 402 (Arf5), and inserted in pCEP4 between *Hind*III and *Bam*HI sites. All constructs were
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29 403 verified by sequencing.

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35 404 **Cell culture.** Huh-7 (Nakabayashi *et al.*, 1982) cells were grown in Dulbecco's modified
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37 405 Eagle's medium (DMEM), high glucose modification, supplemented with glutamax-I and
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39 406 10% FCS.

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42 407 **HCVcc.** The virus JFH1-CSN6A4 used in this study was based on JFH1, and contained cell
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44 408 culture adaptive mutations (Delgrange *et al.*, 2007) and a reconstituted A4 epitope in E1,
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46 409 as previously described (Goueslain *et al.*, 2010). The plasmid pJFH1-CSN6A4 was
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48 410 linearized with *Xba*I and treated with the Mung Bean Nuclease (New England Biolabs).
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50 411 In vitro transcripts were generated using the Megascript kit according to the
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52 412 manufacturer's protocol (Ambion). Ten micrograms of in vitro transcribed RNA were
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54 413 delivered into Huh-7 cells by electroporation as described (Kato *et al.*, 2003). For virus
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56 414 production, electroporated cells were passaged 3 days after electroporation and grown
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3 415 to confluence. The culture medium was collected every day, titrated, aliquoted and
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5 416 stored at -80°C. For infection assays, sub-confluent naïve Huh-7 cells grown in a P24
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7 417 well were incubated with 50 µl of this virus preparation diluted to 200 µl of medium for
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9 418 2 hours (M.O.I. ~0.5), and the inoculate was replaced with fresh culture medium. In
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11 419 order to reach near 100% infected cells in experiments with Arf1 mutants, cells were
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13 420 infected with 200 µl of undiluted HCVcc stock with a higher titre, (M.O.I. ~5). In all
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15 421 experiments, infections were scored at 30 hpi.
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19 422 **Adenovirus.** A recombinant defective adenovirus expressing a green fluorescent
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21 423 protein (EGFP) was as previously described (Farhat *et al.*, 2013). Cells were infected for
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23 424 1 hour at 37°C, and fixed for 20 minutes with PFA 3% at 16 hours post-infection.
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26 425 **DNA transfection.** Twenty-four hours before transfection, cells were seeded in 24-well
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28 426 clusters to reach ~70% confluence the next day. Cells were transfected with 0.5 µg of
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30 427 plasmid DNA mixed with Trans-IT LT1 reagent following the instructions of the
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32 428 manufacturer (Mirus). Cells transfected with pCEP4-based constructs were co-
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34 429 transfected with pPUR (clontech) at a 1:20 ratio, and selected for with 5 µg/ml
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36 430 puromycin for 3 days, and cultured with no puromycin for 4 days before siRNA
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38 431 transfection.
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43 432 **RNA interference.** RNA interference experiments were carried out with pools of four
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45 433 different synthetic double-stranded siRNAs to the same target (on-target plus smart
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47 434 pool reagents from Dharmacon). Due to an off-target effect with the pool against Arf1 we
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49 435 used a mix of 2 individual siRNAs (J-011580-05-0005 & J-011580-08-0005). The control
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51 436 used in this study was the on-target plus non-targeting siRNA #1 (D-001810-01-20).
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54 437 For siRNA transfection, 3 µl of lipofectamine RNAi MAX (Life Technologies) were added
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56 438 to 0.5 ml of D-PBS and incubated for 3 min. In a 6-well plate, 2.5 µl of siRNA at 20µM
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58 439 were spotted in the center of a well. In case of double siRNA transfection, 1.25 µl of each
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3 440 siRNA was used. Then, the diluted transfection reagent was added to the siRNA and the
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5 441 mixture was incubated for 30 min at room temperature. At the end of this incubation,
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7 442 2.5×10^5 freshly trypsinized cells in a volume of 2 ml of culture medium were added to the
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9 443 transfection mix and the cells were returned to 37°C. Cells were trypsinized 24 h later
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11 444 and seeded on glass cover slips, and analyzed by immunofluorescence 3 or 4 days after
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13 445 siRNA transfection. For quantifying HCV infection, siRNA-treated cells were infected 48
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15 446 h after trypsinization. Just before infection, extra wells of cells treated with each siRNA
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17 447 were used to extract RNA for quantifying the depletion efficiency. Infected cells were
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19 448 stained with anti-E1 mAb A4 and DAPI at 30 hpi and HCV-infected were counted. At
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21 449 least 5000 cells were counted per experiment for each condition.
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26 450 **Immunoblotting.** Cells were rinsed 3 times with cold PBS, and lysed at 4°C for 20 min
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28 451 in a buffer containing 50 mM TrisCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1% Triton- X,
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30 452 0.1% SDS, 1 mM PMSF, and a mix of protease inhibitors (Complete). Insoluble material
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32 453 was removed by centrifugation at 4°C. The protein content was determined by the
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34 454 bicinchoninic acid method as recommended by the manufacturer (Sigma), using bovine
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36 455 serum albumin as the standard. The proteins were then resolved by SDS-PAGE and
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38 456 transferred onto nitrocellulose membranes (Hybond-ECL; Amersham) using a Trans-
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40 457 Blot apparatus (Bio-Rad). Proteins of interest were revealed with specific primary
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42 458 antibodies, followed by species-specific secondary antibodies conjugated to peroxidase.
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44 459 Proteins were visualized using enhanced chemiluminescence (ECL Plus, GE healthcare).
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46 460 The signals were recorded using a LAS 3000 apparatus (Fujifilm). Quantification of
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48 461 unsaturated signals was carried out using the gel quantification function of ImageJ.
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53 462 **Immunofluorescence microscopy.** Indirect immunofluorescence labeling was
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55 463 performed as previously described (Rouillé *et al.*, 2006). Lipid droplets were stained
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57 464 with BODIBY 493/503 (0.5 µg/ml; Invitrogen) for 10 min at room temperature. Nuclei
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3 465 were stained with DAPI. For colocalization experiments, confocal microscopy was
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5 466 carried out with an LSM780 confocal microscope (Zeiss) using a 63X oil immersion
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7 467 objective with a 1.4 numerical aperture. Signals were sequentially collected using single
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9 468 fluorescence excitation and acquisition settings to avoid crossover. Images were
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11 469 processed using Adobe Photoshop software CS4.

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14 470 **BFA rescue experiments.** Cells were transfected with GBF1 mutants and then infected
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16 471 with JFH1-CSN6A4 48 h post transfection in the presence of 50 ng/ml of BFA, or the
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18 472 corresponding volume of ethanol (BFA solvent). Cells were lysed at 30 hpi and the
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20 473 expression levels of HCV E1 (A4) were measured by Western blot. The expression level
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22 474 of each mutant with ethanol was set to 100%. The relative expression of HCV E1 of the
23
24 475 BFA treated samples was normalized to the ethanol control.

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28 476 **Secretion assays.** Sub-confluent cell cultures grown in 12-well plates were incubated
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30 477 for 24 h in 1 ml of complete culture medium. Culture media were collected and
31
32 478 centrifuged to remove cells debris. Cells were rinsed with PBS, and lysed for 20 min on
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34 479 ice. The HSA concentration in the supernatants and lysates was determined by ELISA,
35
36 480 using human serum albumin (HSA) as a standard, as described (Snooks *et al.*, 2008).
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38 481 Apolipoprotein E was quantified using a commercial ELISA kit from Mabtech. The
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40 482 percentage of secretion was calculated as the percentage of HSA/apoE in the medium
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42 483 divided by the total amount of HSA/apoE in the medium and the lysate.

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47 484 **Replication assay.** The construct used for the replication assay (HCVcc-Rluc/ Δ E1E2)
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49 485 was as previously described (Goueslain *et al.*, 2010). Huh-7 cells were electroporated
50
51 486 with HCVcc-Rluc/ Δ E1E2 in vitro transcribed RNA and seeded in 24-well plates. The
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53 487 luciferase activity was measured 4 h, 24 h, 48 h and 72 h post-electroporation using the
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55 488 *Renilla* luciferase assay system kit from Promega.

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3 489 **Quantitative RT-PCR.** Total RNA was extracted from siRNA-transfected and control
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5 490 cells using Nucleospin RNA II extraction kit (Macherey-Nagel), which includes a DNase I
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7 491 treatment. cDNA was obtained from RNA using the High Capacity cDNA Reverse
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9 492 transcription kit (Life Technologies) in a final volume of 20 μ l. Quantitative RT-PCR
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11 493 analysis was performed using the Taqman® pre-designed gene expression assay
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13 494 approach (Applied Biosystems), using 1 μ l of cDNA and premade probes designed by the
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15 495 manufacturer. The ratio of the mRNA level of each gene to that of large ribosomal
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17 496 protein P0 (RPLP0) endogenous control mRNA was calculated by the $\Delta\Delta$ Ct method
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19 497 (Livak and Schmittgen, 2001), and a value of 100 was assigned to control siRNA-
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21 498 transfected cells. Each experiment was performed in triplicate and repeated three times.
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26 499 **HCVpp.** The luciferase-based HCV-pseudotyped retroviral particles (HCVpp) infection
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28 500 assay was performed as previously described (Op De Beeck *et al.*, 2004).
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38
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45
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49 509 The authors have no conflict of interest to declare.
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3 693 **FIGURES LEGENDS**
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8 **Figure 1. Activity of GBF1 truncation mutants in HCV replication.** (A) Schematic
9 representation of GBF1 deletion constructs. Conserved domains are indicated in grey
10 and black. (B) Huh-7 cells were transfected with the indicated constructs, infected 16 h
11 later in the presence or the absence of BFA (50 ng/ml) and lysed 30 hpi. BFA was added
12 during the 2-h infection and was present throughout the experiment. Cell lysates were
13 analyzed by immunoblotting with antibodies to E1 (top), GFP (middle) or tubulin
14 (bottom). (C) Quantification of E1 signals. For each construct, the expression of E1 in the
15 presence of BFA is expressed as a percentage of its expression in the absence of BFA.
16 Values are means \pm SD of 3 independent experiments. (D) Transfected Huh-7 cells were
17 incubated for 24 h with 50 ng/ml BFA and the amounts of HSA in cell lysates and culture
18 media were quantified by ELISA. Values are means \pm SD of 3 independent experiments
19 and are expressed as percentage of secretion. -BFA, mock-transfected cells incubated in
20 the absence of BFA.
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39 **Figure 2. Effect of T31N and Q71L of Arf1 mutants on HCV infection.** (A) Huh-7 cells
40 were transfected with plasmids expressing Arf1-GFP (WT), Arf1 T31N-GFP or Arf1
41 Q71L-GFP, and infected with HCV 16 hours after transfection. The cells were fixed at 30
42 hpi and infected cells were labeled with an anti-E1 antibody (red). Cells expressing Arf1
43 constructions were detected by GFP fluorescence (green). Each field is presented in
44 duplicate, with an image corresponding to the infection at the top and the image
45 corresponding to merged signals at the bottom, to facilitate the visualization of the red
46 staining. Arrows indicate cells expressing fluorescent fusion proteins. (B) Huh-7 cells
47 expressing GFP or mCherry (FP) or Arf1 constructs fused to GFP or mCherry were
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3 718 infected with HCV and the infection was quantified in at least 100 fluorescent cells per
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5 719 experiment for each construct (black series). For mock-transfected cells, the infection in
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7 720 the total population was quantified. A similar analysis was performed with an
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9 721 adenovirus expressing GFP in cells expressing the mCherry constructs (grey series). The
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11 722 results are averages of 3 independent experiments (\pm SD), * $P < 0.05$, *** $P < 0.001$.
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16 724 **Figure 3. Impact of Arf proteins depletion on HCV replication.** (A) Huh-7 cells were
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18 725 transfected with indicated siRNA, infected with HCV or adenovirus at 72 h post
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20 726 transfection, fixed at 30 hpi and processed for detection of infected cells by
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22 727 immunofluorescence. Infection of non-targeting siRNA-treated samples is expressed as
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24 728 100% (*** $P < 0.001$, 1-way ANOVA). (B) Total RNA was extracted from siRNA-
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26 729 transfected cells at 72 h post transfection, and the indicated mRNAs were quantified by
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28 730 RT-qPCR. mRNA amounts in non-targeting siRNA-treated samples are expressed as
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30 731 100%. (C) pCEP4, pCEP4-Arf4m or pCEP4-Arf5m (both from mouse) transfected Huh-7
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32 732 cells were transfected with indicated siRNA and analyzed by immunoblotting at 3 days
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34 733 post transfection using Arf4, Arf5 and tubulin antibodies. Note that the anti-Arf4
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36 734 antibody does not detect the transfected murine Arf4. (D) pCEP4, pCEP4-Arf4m or
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38 735 pCEP4-Arf5m-transfected Huh-7 cells were transfected with indicated siRNA and
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40 736 infected with HCV 3 days later. Cells were fixed at 30 hpi, labeled with an anti-E1
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42 737 antibody and the number of infected cells was counted. The number of infected cells in
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44 738 pCEP4/non-targeting siRNA-transfected cells was expressed as 100%. Error bars
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46 739 represent standard error of the means (SEM) from 4 independent experiments (**
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48 740 $P < 0.01$, *** $P < 0.001$, pCEP4-Arf vs pCEP4, 2-way ANOVA). (E) Huh-7 cells were
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50 741 transfected with indicated siRNAs and electroporated 3 days post transfection with a
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52 742 recombinant HCV genome containing a deletion in E1E2 and expressing *Renilla*
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3 743 luciferase. Samples were harvested for luciferase assay at 4, 24, 48, and 72 h post
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5 744 electroporation. Error bars indicate standard errors of the mean for 3 independent
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7 745 experiments performed in triplicate.
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11 747 **Figure 4. Impact of Arf pair depletion on the secretory pathway.** (A) Huh-7 cells
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14 748 transfected with the indicated siRNA were seeded in 12-well plates, and cultured for 24
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16 749 h. The amounts of human serum albumin (HSA) and of apolipoprotein E (apoE) in the
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18 750 conditioned culture media and in cell lysates were quantified with an ELISA assay and
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20 751 expressed as percentage of secretion. Error bars represent standard deviation of 3
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22 752 independent experiments performed in duplicate (***) $P < 0.001$, 1-way ANOVA). (B) Huh-
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25 753 7 were transfected with indicated siRNAs and fixed 72 h later. Cells were fixed and
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27 754 processed for immunofluorescent detection of ERGIC53, GBF1, GM130 and TGN46
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29 755 (white). Nuclei were stained with DAPI (blue). Representative confocal images are
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32 756 shown. Bar, 20 μm .
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37 758 **Figure 5. Impact of Arf4 and Arf5 depletion on lipid droplets.** (A) Huh-7 cells were
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39 759 transfected with indicated siRNAs and fixed 72 h later. Cells were fixed and processed
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41 760 for immunofluorescent detection of GM130 (red). Lipid droplets were stained with
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43 761 BODIBY 493/503 (green) and nuclei with DAPI (blue). (B) Huh-7 cells were cultured for
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45 762 24 h in the presence or the absence of BFA (50 ng/ml), and processed for the detection
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47 763 of lipid droplets and nuclei. Representative confocal images are shown. Bars, 20 μm .
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53 765 **LEGENDS TO THE SUPPLEMENTARY FIGURES**

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3 767 **Figure S1. Off-target effect of Arf1 siRNA pool.** Huh-7 cells were transfected with
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5 768 indicated siRNA and total RNA was extracted 24 h post transfection. Arf1 and Arf3
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7 769 mRNAs were quantified by RT-qPCR and mRNA amounts in non-targeting siRNA-treated
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9 770 samples are expressed as 100%.

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13 772 **Figure S2. Impact of class II Arf proteins depletion on HCVpp, VSV-Gpp and**
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16 773 **RD114pp entry.** Huh-7 cells were transfected with indicated siRNA and infected with
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18 774 HCVpp, VSV-Gpp or RD114pp 3 days post transfection. Samples were harvested for
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20 775 luciferase assay at 48 hpi. Error bars indicate standard deviation for 3 independent
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22 776 experiments performed in triplicate.

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28 778 **Figure S3. Immunofluorescence analysis of Arf4 and Arf5 intracellular localization**
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30 779 **in non infected Huh-7 cells.** Huh-7 cells transfected with Arf4-GFP or Arf5-GFP
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32 780 expression plasmids were processed for immunofluorescent detection of GM130 (A) or
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34 781 GBF1 (B). Representative confocal images of transfected cells are shown together with
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36 782 the merge image. Bars, 20 μ m.

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41 784 **Figure S4. Immunofluorescence analysis of Arf4 and Arf5 intracellular localization**
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44 785 **in infected Huh-7 cells.** Huh-7 cells infected with HCV and transfected with Arf4-GFP or
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46 786 Arf5-GFP expression plasmids were processed for immunofluorescent detection of NS3
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48 787 (A) or NS5A (B). Representative confocal images of transfected cells are shown together
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50 788 with the merge image. Bars, 20 μ m.

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55 790 **Figure S5. Impact of the depletion of different Arf pairs on lipid droplets.** (A) Huh-7
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57 791 were transfected with indicated siRNAs and fixed 72 h later. Cells were fixed and

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3 792 processed for immunofluorescent detection of GM130 (red). Lipid droplets were stained
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5 793 with BODIPY 493/503 (green) and nuclei with DAPI (blue). Representative confocal
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7 794 images are shown.
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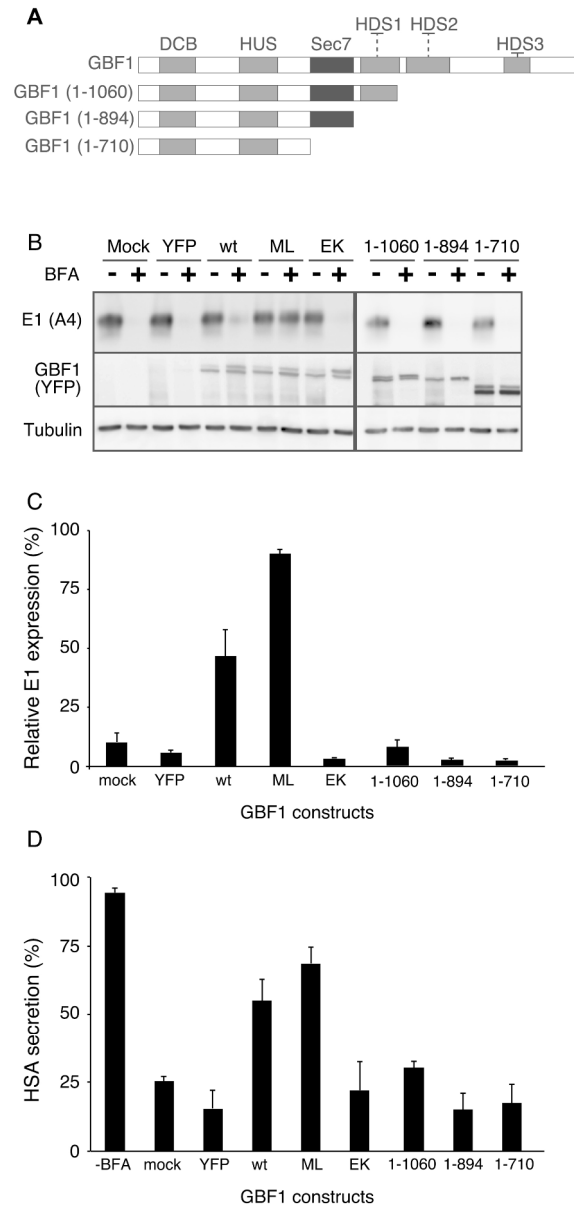


Figure 1. Activity of GBF1 truncation mutants in HCV replication. (A) Schematic representation of GBF1 deletion constructs. Conserved domains are indicated in grey and black. (B) Huh-7 cells were transfected with the indicated constructs, infected 16 h later in the presence or the absence of BFA (50 ng/ml) and lysed 30 hpi. BFA was added during the 2-h infection and was present throughout the experiment. Cell lysates were analyzed by immunoblotting with antibodies to E1 (top), GFP (middle) or tubulin (bottom). (C) Quantification of E1 signals. For each construct, the expression of E1 in the presence of BFA is expressed as a percentage of its expression in the absence of BFA. Values are means \pm SD of 3 independent experiments. (D) Transfected Huh-7 cells were incubated for 24 h with 50 ng/ml BFA and the amounts of HSA in cell lysates and culture media were quantified by ELISA. Values are means \pm SD of 3 independent experiments and are expressed as percentage of secretion. -BFA, mock-transfected cells incubated in the absence of BFA.

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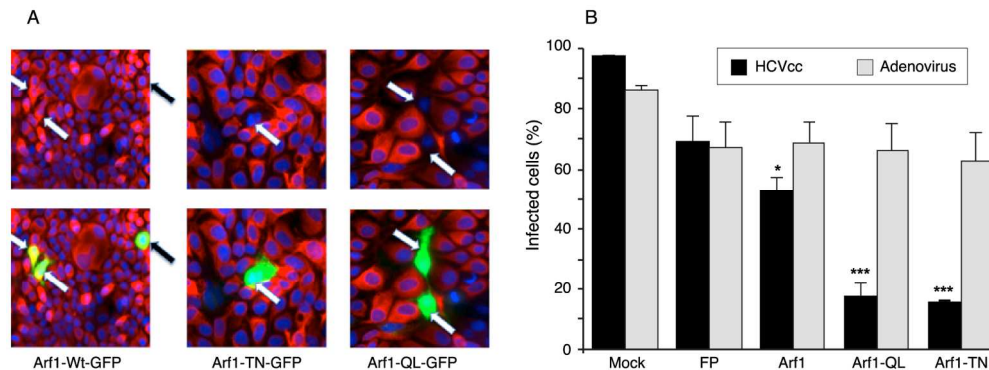


Figure 2. Effect of T31N and Q71L of Arf1 mutants on HCV infection. (A) Huh-7 cells were transfected with plasmids expressing Arf1-GFP (WT), Arf1 T31N-GFP or Arf1 Q71L-GFP, and infected with HCV 16 hours after transfection. The cells were fixed at 30 hpi and infected cells were labeled with an anti-E1 antibody (red). Cells expressing Arf1 constructions were detected by GFP fluorescence (green). Each field is presented in duplicate, with an image corresponding to the infection at the top and the image corresponding to merged signals at the bottom, to facilitate the visualization of the red staining. Arrows indicate cells expressing fluorescent fusion proteins. (B) Huh-7 cells expressing GFP or mCherry (FP) or Arf1 constructs fused to GFP or mCherry were infected with HCV and the infection was quantified in at least 100 fluorescent cells per experiment for each construct (black series). For mock-transfected cells, the infection in the total population was quantified. A similar analysis was performed with an adenovirus expressing GFP in cells expressing the mCherry constructs (grey series). The results are averages of 3 independent experiments (\pm SD), * $P < 0.05$, *** $P < 0.001$.

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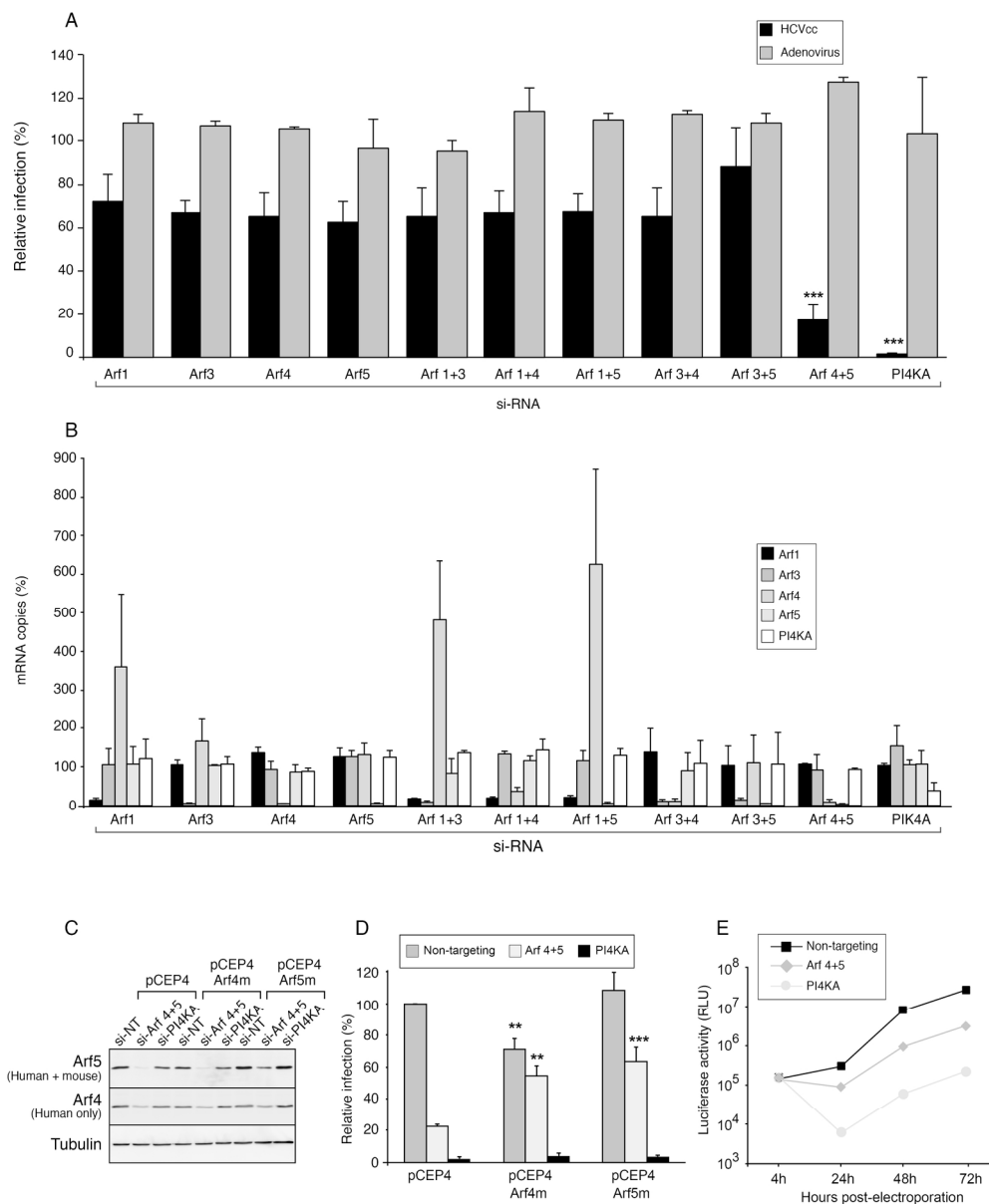


Figure 3. Impact of Arf proteins depletion on HCV replication. (A) Huh-7 cells were transfected with indicated siRNA, infected with HCV or adenovirus at 72 h post transfection, fixed at 30 hpi and processed for detection of infected cells by immunofluorescence. Infection of non-targeting siRNA-treated samples is expressed as 100% (***) $P < 0.001$, 1-way ANOVA). (B) Total RNA was extracted from siRNA-transfected cells at 72 h post transfection, and the indicated mRNAs were quantified by RT-qPCR. mRNA amounts in non-targeting siRNA-treated samples are expressed as 100%. (C) pCEP4, pCEP4-Arf4m or pCEP4-Arf5m (both from mouse) transfected Huh-7 cells were transfected with indicated siRNA and analyzed by immunoblotting at 3 days post transfection using Arf4, Arf5 and tubulin antibodies. Note that the anti-Arf4 antibody does not detect the transfected murine Arf4. (D) pCEP4, pCEP4-Arf4m or pCEP4-Arf5m-transfected Huh-7 cells were transfected with indicated siRNA and infected with HCV 3 days later. Cells were fixed at 30 hpi, labeled with an anti-E1 antibody and the number of infected cells was counted. The number of infected cells in pCEP4/non-targeting siRNA-transfected cells was expressed as 100%. Error bars represent standard error of the means (SEM) from 4 independent experiments (** $P < 0.01$, *** $P < 0.001$, pCEP4-Arf vs pCEP4, 2-way

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3 ANOVA). (E) Huh-7 cells were transfected with indicated siRNAs and electroporated 3 days post transfection
4 with a recombinant HCV genome containing a deletion in E1E2 and expressing Renilla luciferase. Samples
5 were harvested for luciferase assay at 4, 24, 48, and 72 h post electroporation. Error bars indicate standard
6 errors of the mean for 3 independent experiments performed in triplicate.
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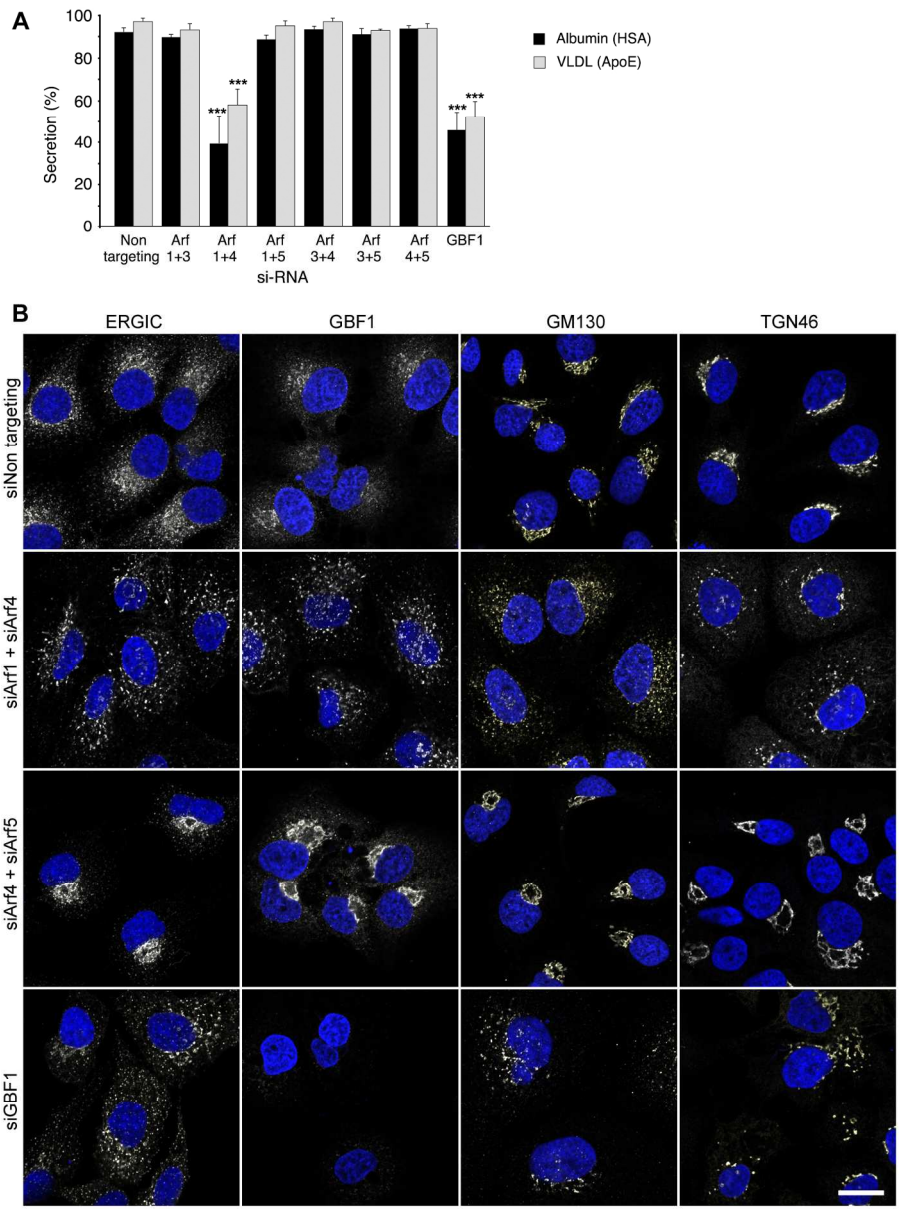


Figure 4. Impact of Arf pair depletion on the secretory pathway. (A) Huh-7 cells transfected with the indicated siRNA were seeded in 12-well plates, and cultured for 24 h. The amounts of human serum albumin (HSA) and of apolipoprotein E (apoE) in the conditioned culture media and in cell lysates were quantified with an ELISA assay and expressed as percentage of secretion. Error bars represent standard deviation of 3 independent experiments performed in duplicate (***) P<0.001, 1-way ANOVA). (B) Huh-7 were transfected with indicated siRNAs and fixed 72 h later. Cells were fixed and processed for immunofluorescent detection of ERGIC53, GBF1, GM130 and TGN46 (white). Nuclei were stained with DAPI (blue). Representative confocal images are shown. Bar, 20 μm.
157x211mm (300 x 300 DPI)

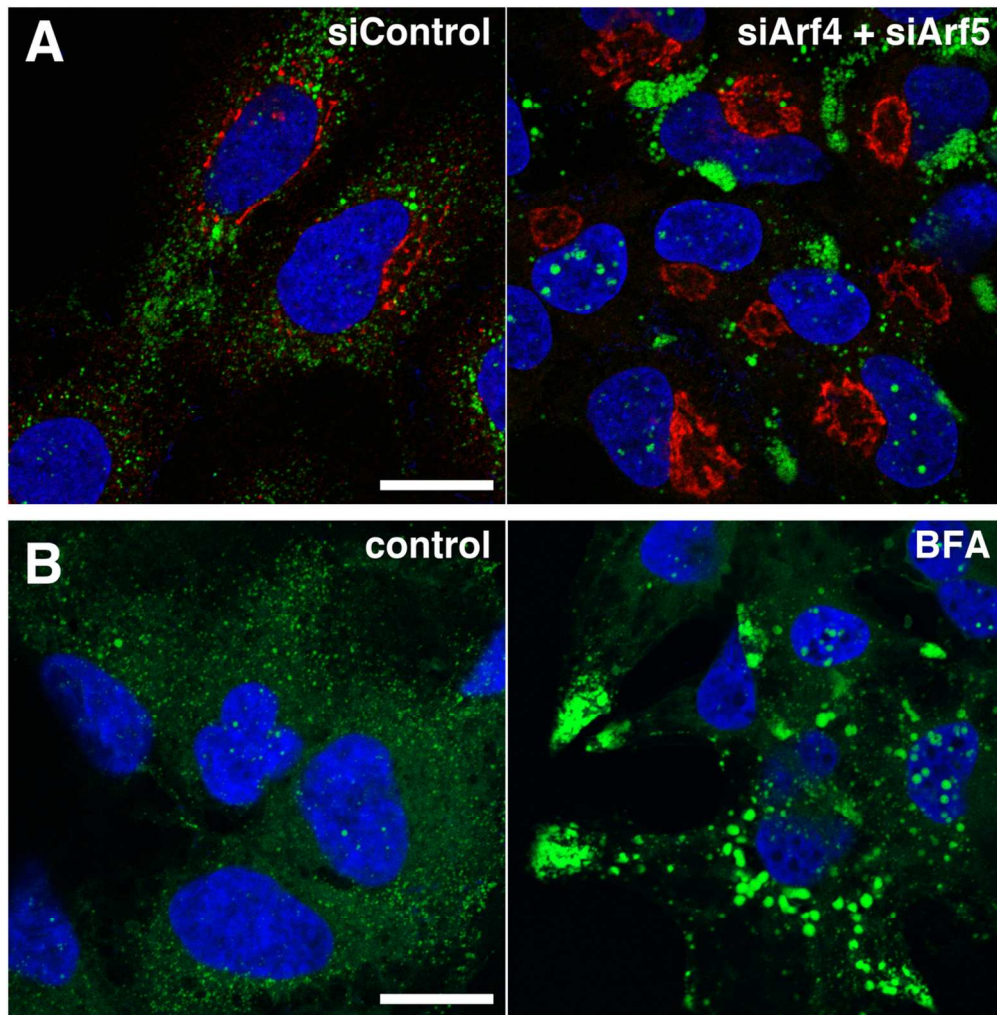


Figure 5. Impact of Arf4 and Arf5 depletion on lipid droplets. (A) Huh-7 cells were transfected with indicated siRNAs and fixed 72 h later. Cells were fixed and processed for immunofluorescent detection of GM130 (red). Lipid droplets were stained with BODIBY 493/503 (green) and nuclei with DAPI (blue). (B) Huh-7 cells were cultured for 24 h in the presence or the absence of BFA (50 ng/ml), and processed for the detection of lipid droplets and nuclei. Representative confocal images are shown. Bars, 20 μ m.
111x113mm (300 x 300 DPI)