



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

Rayan Farhat, Karin Séron, Juliette Ferlin, Lucie Fénéant, Sandrine Belouzard, Lucie Goueslain, Catherine Jackson, Jean Dubuisson, Yves Rouillé

► To cite this version:

Rayan Farhat, Karin Séron, Juliette Ferlin, Lucie Fénéant, Sandrine Belouzard, et al.. Identification of class II ADP-ribosylation factors as cellular factors required for hepatitis C virus replication. Cellular Microbiology, Wiley, 2016, 18 (8), pp.1121-1133. 10.1111/cmi.12572 . hal-02114450

HAL Id: hal-02114450

<https://hal.archives-ouvertes.fr/hal-02114450>

Submitted on 29 Apr 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



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

Journal:	<i>Cellular Microbiology</i>
Manuscript ID	CMI-15-0214.R2
Manuscript Type:	Research article
Date Submitted by the Author:	n/a
Complete List of Authors:	Farhat, Rayan; Institut Pasteur de Lille, CIIL Séron, Karin; Institut Pasteur de Lille, CIIL Ferlin, Juliette; Institut Pasteur de Lille, Center for Infection & Immunity of Lille Fénéant, Lucie; Institut Pasteur de Lille, CIIL Belouzard, Sandrine; Institut Pasteur de Lille, CIIL Goueslain, Lucie; Institut Pasteur de Lille, CIIL Jackson, Catherine; Institut Jacques Monod, CNRS UMR 7592 Dubuisson, Jean; Institut Pasteur de Lille, CIIL Rouillé, Yves; Institut Pasteur de Lille, Center for Infection & Immunity of Lille
Key Words:	Infection, Membrane, Viruses (phages)

1
2
3 1
4 2
5
6 3 **Identification of class II ADP-ribosylation factors as cellular**
7
8 **factors required for hepatitis C virus replication**
9 4
10 5
11 6

12
13
14 7 **Running title: Class II Arfs and HCV replication**
15 8
16 9

17
18
19 10 Rayan Farhat¹, Karin Séron¹, Juliette Ferlin¹, Lucie Fénéant¹, Sandrine Belouzard¹, Lucie
20 11 Goueslain^{1,2}, Catherine L Jackson², Jean Dubuisson¹, Yves Rouillé^{1*}
21 12

22
23
24 13 ¹Center for Infection & Immunity of Lille, Inserm U1019, CNRS UMR8204, Institut
25 14 Pasteur de Lille, Université Lille Nord de France, Lille, France
26 15

27
28
29 16 ²Institut Jacques Monod, CNRS UMR 7592, Université Paris Diderot, Sorbonne Paris Cité,
30 17 Paris, France
31 18

32
33
34 19 *Corresponding author. E-mail: yves.rouille@ibl.cnrs.fr; Phone: +33-320-87-10-27; Fax:
35 20 +33-320-87-12-01.
36 21
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 22 **SUMMARY**
4

5 23 GBF1 is a host factor required for hepatitis C virus (HCV) replication. GBF1 functions as
6
7 24 a guanine nucleotide exchange factor (GEF) for G-proteins of the Arf family, which
8
9 25 regulate membrane dynamics in the early secretory pathway and the metabolism of
10
11 26 cytoplasmic lipid droplets. Here we established that the Arf-GEF activity of GBF1 is
12
13 27 critical for its function in HCV replication, indicating that it promotes viral replication by
14
15 28 activating one or more Arf family members. Arf involvement was confirmed with the use
16
17 29 of two dominant-negative Arf1 mutants. However siRNA-mediated depletion of Arf1,
18
19 30 Arf3 (class I Arfs), Arf4 or Arf5 (class II Arfs), which potentially interact with GBF1, did
20
21 31 not significantly inhibit HCV infection. In contrast, the simultaneous depletion of both
22
23 32 Arf4 and Arf5, but not of any other Arf pair, imposed a significant inhibition of HCV
24
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
28
29 35 accumulation of lipid droplets. A similar phenotype of lipid droplet accumulation was
30
31 36 also observed when GBF1 was inhibited by brefeldin A. In contrast, the simultaneous
32
33 37 depletion of both Arf1 and Arf4 resulted in secretion inhibition and Golgi scattering, two
34
35 38 actions reminiscent of GBF1 inhibition. We conclude that GBF1 could regulate different
36
37 39 metabolic pathways through the activation of different pairs of Arf proteins.
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

1
2
3 66 conserved domains (Bui *et al.*, 2009). The GEF activity is catalyzed by the Sec7 domain.
4
5 67 Our knowledge of the functions of the other conserved domains of GBF1 is still scarce
6
7 68 (Bui *et al.*, 2009; Belov *et al.*, 2010; Bouvet *et al.*, 2013).
8
9

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;
13
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.
17
18

19 73 It is generally assumed to function as an ArfGEF activating Arf1, which in turn would
20
21 74 recruit the COP-I coatomer, a molecular machinery involved in intracellular transport,
22
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;
26
27 77 Wang *et al.*, 2012). Accordingly, a GBF1-Arf1-COP-I pathway has been proposed to play a
28
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
34
35 81 demonstrated. For instance, in poliovirus infection, the transient recruitment of GBF1 at
36
37 82 replication complexes is not coupled to COP-I recruitment (Richards *et al.*, 2014),
38
39 83 suggesting the existence of distinct functions for GBF1 at viral replication complexes and
40
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
46
47 87 activation by GBF1 of an Arf1 effector different from COP-I, the phosphatidylinositol 4-
48
49 88 kinase III β , has been proposed to be involved in HCV replication (Zhang *et al.*, 2012).
50
51 89 However the involvement of this kinase during HCV replication is still controversial (Tai
52
53 90 *et al.*, 2009; Vaillancourt *et al.*, 2009; Berger *et al.*, 2009; Borawski *et al.*, 2009; Trotard *et*
54
55
56
57
58
59
60

1
2
3 91 *al.*, 2009). Other possibilities for GBF1 function during viral replication include the
4
5 92 activation of other members of the Arf family or mechanisms unrelated to Arf activation.
6
7 93 For example, the mechanism of action of GBF1 during poliovirus replication has been
8
9
10 94 demonstrated not to depend on its catalytic Sec7 domain and therefore on its GEF
11
12 95 activity (Belov *et al.*, 2010). In this study, we investigated the mechanism of action of
13
14 96 GBF1 in HCV replication. Our data support a model in which class II Arf proteins mediate
15
16 97 GBF1 function.
17
18
19 98

99 **RESULTS**

100 ***Role of the Sec7 domain of GBF1 in HCV infection.*** It has recently been reported that
101 GBF1 but not Arf activation is required for poliovirus replication (Belov *et al.*, 2010),
102 suggesting that GBF1 may have other functions than Arf activation during a viral
103 infection. More specifically, a catalytically inactive GBF1 truncation mutant lacking the
104 Sec7 domain has been reported to be sufficient for rescuing poliovirus replication from
105 BFA inhibition. To investigate if GBF1 could also function in a Sec7-independent manner
106 in HCV replication, we expressed a series of GBF1 truncation mutants (figure 1A) in
107 Huh-7 cells and infected them with HCV in the presence of BFA. We used a dose of 50
108 ng/ml BFA, which decreases HCV infection about 10 times and has minimal cytotoxic
109 effects in Huh-7 cells during the time scale of the HCV infection assay (Goueslain *et al.*,
110 2010). We previously showed that BFA has no impact on HCV entry and inhibits the
111 replication step of the HCV life cycle (Goueslain *et al.*, 2010). GBF1 construct expression
112 and HCV infection were probed by immunoblotting at 30 hpi. At this time, only cells
113 initially infected are positive (Afzal *et al.*, 2014), implying that the impact of BFA
114 inhibition of progeny virion release on HCV protein expression is negligible.

1
2
3 115 BFA treatment had no impact on the expression of GBF1 constructs (figure 1B). In the
4
5 116 absence of BFA, similar E1 expression levels were observed with all GBF1 constructs,
6
7 117 indicating that none of these constructs had any dominant-negative effect on HCV
8
9 118 infection. In the presence of BFA, E1 expression levels were reduced about 10-20 times
10
11 119 in untransfected cells or in cells expressing YFP. HCV infection was partially rescued in
12
13 120 cells expressing full-length wild-type or M832L BFA-resistant constructs, whereas the
14
15 121 E794K inactive mutant was unable to rescue HCV infection in the presence of BFA, as
16
17 122 previously reported (Goueslain *et al.*, 2010). Importantly, unlike for poliovirus, all the C-
18
19 123 terminal deletion constructs including GBF1(1-710), which lacks a Sec7 domain, were
20
21 124 unable to rescue HCV infection in these complementation experiments (figure 1B and C).
22
23 125 Therefore, these results support a Sec7-dependent function of GBF1 in HCV infection.
24
25 126 As a control, we also quantified the impact of GBF1 constructs on the secretion of human
26
27 127 serum albumin (HSA) by BFA-treated Huh-7 cells. A well-established function of GBF1 is
28
29 128 the control of membrane dynamics in the early secretory pathway, and this function
30
31 129 relies on Arf activation. Therefore, HSA secretion is a marker of GBF1-mediated Arf
32
33 130 activation in these experiments. The treatment of Huh-7 cells with 50 ng/ml BFA
34
35 131 decreased albumin secretion in control mock-, or YFP-transfected cells (figure 1D). As
36
37 132 expected, wild type and M832L GBF1 constructs partially restored albumin secretion,
38
39 133 but none of the other constructs did. These results indicate a similar requirement of
40
41 134 GBF1 domains for HCV infection and for Arf activation and are consistent with a
42
43 135 requirement of ArfGEF activity to promote HCV infection.
44
45
46
47
48
49
50

51 136

52
53 137 ***Arf1 dominant negative mutants inhibit HCV infection.*** Our results with GBF1
54
55 138 truncation mutants suggested that the activation of at least one BFA-sensitive Arf family
56
57 139 member is required for HCV infection. We further investigated this question using two
58
59
60

1
2
3 140 different mutants of Arf1 affecting either GDP exchange (T31N) or GTP hydrolysis
4
5 141 (Q71L). When overexpressed, these two constructs act as dominant negative mutants in
6
7 142 a protein transport assay and block the intracellular traffic at different steps of the early
8
9 143 secretory pathway (Dascher and Balch, 1994). Arf mutants were expressed as EGFP or
10
11 144 mCherry fusion proteins in Huh-7 cells and the cells were infected with HCV. The
12
13 145 infection was monitored by immunofluorescence (figure 2A) and the percentage of
14
15 146 fluorescent cells infected was quantified. As expected, the expression of the T31N
16
17 147 mutant, which has a BFA-like effect, strongly inhibited HCV infection (figure 2B). The
18
19 148 Q71L mutant, which is locked in the active, GTP-bound form, also strongly inhibited HCV
20
21 149 infection, and the wild type Arf1 construct had a moderate impact compared to EGFP
22
23 150 and mCherry controls. On the other hand, Arf1 constructs did not have any effect on
24
25 151 adenovirus infection, indicating that the effects observed with HCV did not result from
26
27 152 any cytotoxic effects of Arf1 constructs. These results confirmed the involvement of an
28
29 153 Arf family member in HCV infection.
30
31
32
33
34
35

36
37 155 ***Role of Arf family members in HCV infection.*** The results with dominant negative
38
39 156 mutants of Arf1 suggest that Arf1 and/or other Arf family members are involved in HCV
40
41 157 infection. We used siRNA technology to determine which Arf proteins are specifically
42
43 158 involved in HCV infection. Given the BFA sensitivity of HCV infection, we focused our
44
45 159 study on class-I and class-II Arfs. Arf1, Arf3, Arf4 and Arf5 (human cells have no Arf2)
46
47 160 were targeted with pools of 4 siRNAs, except for Arf1, which was targeted by 2 siRNAs
48
49 161 only, because we found that the commercial Arf1 pool contains 2 individual siRNAs also
50
51 162 targeting Arf3 (supplementary figure 1). Surprisingly, the depletion of each Arf protein
52
53 163 resulted in a moderate (30-35%) inhibition of HCV infection (figure 3A). To assess the
54
55 164 extent of siRNA-mediated Arf depletion, we quantified mRNAs by quantitative RT-PCR.
56
57
58
59
60

1
2
3 165 The depletions were specific and reached 87% inhibition for Arf1 and about 95%
4
5 166 inhibition for the other Arfs (figure 3B). We also observed an up-regulation of Arf4
6
7 167 expression upon Arf1 and to a lesser extent Arf3 depletion, suggesting the existence of
8
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
12
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

19 173 observed upon Arf1+Arf3 depletion or Arf1+Arf5 depletion, confirming the up-

21 174 regulation of Arf4 in Arf1-depleted cells (figure 3B). Interestingly, HCV infection was

23 175 decreased down to 17±8% in cells depleted of both Arf4 and Arf5, whereas the

25 176 depletion of other pairs did not significantly decrease more HCV infection than single Arf

27 177 depletions (figure 3A). These results indicate a special importance of class-II Arfs in HCV

29 178 infection.

31 179 To rule out off-target effects, Arf4 or Arf5 were expressed in siRNA-treated cells. Arf

33 180 proteins of murine origin were expressed in Huh-7 cells simultaneously depleted of both

35 181 Arfs. Immunoblot analysis confirmed Arf5 expression (figure 3C). However, Arf4

37 182 expression could not be confirmed due to the lack of reactivity toward murine Arf4 of

39 183 the antibody. HCV infection was partially restored in Arf4-expressing cells and in Arf5-

41 184 expressing cells (figure 3D). We also observed a slight decrease of HCV infection in cells

43 185 transfected with Arf4 in the control condition (figure 3D). The expression of Arf4 or Arf5

45 186 in double-depleted cells increased HCV infection up to levels similar to those observed

47 187 in cells depleted of a single Arf protein (figure 3A). In contrast, no rescue of HCV

49 188 infection was observed in cells transfected with siRNAs to PI4KA, as expected (figure

51 189 3D).

1
2
3 190 To determine if the replication step of the HCV life cycle is affected by the simultaneous
4
5 191 depletion of class-II Arfs, siRNA-transfected Huh-7 cells were electroporated with an in
6
7 192 vitro-transcribed recombinant $\Delta E1E2$ JFH1 genomic RNA expressing a *Renilla* luciferase
8
9 193 reporter. Measuring luciferase activity over a 72-h time course assessed replication.
10
11 194 Luciferase activity was not inhibited at 4h post electroporation, and was reduced about
12
13 195 9 times in cells depleted of Arf4 and Arf5 at 48 and 72 h post electroporation (figure 3E),
14
15 196 indicating an inhibition of HCV replication with no impact on the initial translation level
16
17 197 of HCV RNA.

18
19
20
21 198 We also investigated HCV entry in Huh-7 cells simultaneously depleted of both class II
22
23 199 Arfs using HCVpp. As controls, we used VSV-Gpp, which rely on endocytosis and pH-
24
25 200 dependent fusion for entry, like HCVpp, and RD114pp, which enter by a pH-independent
26
27 201 mechanism. All pseudoparticles were similarly inhibited by the double depletion of Arf4
28
29 202 and Arf5 (supplementary figure 2), indicating a post-fusion inhibition of pseudoparticle
30
31 203 entry. Therefore, we cannot conclude on the function of class II Arfs during the entry
32
33 204 step of the HCV life cycle.
34
35

36
37 205

38
39 206 ***Class II Arfs are not recruited in HCV replication complexes.*** The intracellular
40
41 207 localization of Arf4 and Arf5 in Huh-7 cells was analyzed by immunofluorescence
42
43 208 confocal microscopy. Cells were transfected with expression vectors for Arf4-GFP or
44
45 209 Arf5-GFP. Both Arf4-GFP and Arf5-GFP were observed in Golgi-like perinuclear
46
47 210 structures together with a diffuse staining of the cytosol and the nucleus
48
49 211 (supplementary figure 3), which is consistent with the dual localization of Arf proteins
50
51 212 as membrane-associated and soluble proteins. Arf4- and Arf5-positive perinuclear
52
53 213 structures were also labeled with an antibody to GM130 (supplementary figure 3A),
54
55 214 indicating that they are localized in the cis-Golgi. Interestingly, Arf4 and Arf5
56
57 215 perinuclear structures also colocalized with GBF1 (supplementary figure 3B).
58
59
60

1
2
3 216 In infected cells, the localization of Arf4-GFP and Arf5-GFP was compared to that of NS3
4
5 217 and NS5A, two markers of HCV replication complexes. We did not observe any major
6
7 218 change of Arf4 and Arf5 patterns (supplementary figure 4), except in Arf4-GFP
8
9 219 expressing cells, where the Golgi appears less structured. This tendency toward a less
10
11 220 compact Golgi was also observed in non-infected cells (supplementary figure 3). Very
12
13 221 few levels of colocalization of Arfs and NS3 (supplementary figure 4A) or NS5A
14
15 222 (supplementary figure 4B) were observed, indicating that class II Arfs are not
16
17 223 permanently recruited to HCV replication complexes.
18
19
20
21
22

23 225 ***Impact of Arfs depletion on the secretory pathway.*** To get insight into a mechanism of
24
25 226 action of the pair of class-II Arfs, we analyzed their involvement in the regulation of
26
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
30
31 229 and constitutively secreted by Huh-7 cells. Albumin and apoE secretion were inhibited
32
33 230 in cells with reduced levels of both Arf1 and Arf4, but were not affected by the depletion
34
35 231 of any other pair of Arfs, including Arf4 and Arf 5 (figure 4A). These results indicate that
36
37 232 the simultaneous depletion of class II Arfs has no functional impact on the secretory
38
39 233 pathway.
40
41
42

43 234 To further examine the impact of their depletion on the secretory pathway, we also
44
45 235 investigated the morphology of cellular compartments. We observed a more compact
46
47 236 Golgi morphology in cells simultaneously depleted of Arf4 and Arf5 than in control cells
48
49 237 using the cis-Golgi marker GM130 (figure 4B). In contrast, the depletion of Arf1 and Arf4
50
51 238 resulted in a fragmentation of the cis-Golgi, as previously reported for HeLa cells
52
53 239 (Volpicelli-Daley *et al.*, 2005). A similar scattered Golgi phenotype was observed upon
54
55 240 GBF1 depletion (figure 4B) or after a BFA treatment (data not shown). We did not
56
57
58
59
60

1
2
3 241 observe any morphological change in cells depleted of other pairs of Arfs
4
5 242 (supplementary figure 5). A compaction of the Golgi complex was also observed in class
6
7 243 II Arfs-depleted cells with other markers. In these cells, GBF1 and ERGIC53, two proteins
8
9 244 located both in the cis-Golgi and the ERGIC, and TGN46, a marker of the trans-Golgi
10
11 245 network, all showed a more compact localization (figure 4B), indicating that the effect is
12
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
16
17 248 produced similar phenotypes of scattered or compacted patterns with any of the
18
19 249 markers (data not shown). These results indicate that the pair of class II Arfs does not
20
21 250 contribute to the Golgi scattering effect of GBF1 inhibition.
22
23
24
25
26
27

251

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

1
2
3 266 enlarged lipid droplets. Lipid droplets were very similar to controls for all depletions
4
5 267 except for the pair Arf1 and Arf4, and to some extent the pair Arf3 and Arf4, the
6
7 268 depletion of which resulted in a reduced number of enlarged lipid droplets
8
9
10 269 (supplementary figure 5). This result suggests that the pair of class II Arfs could
11
12 270 participate in GBF1-mediated regulation of lipid metabolism.
13

14 271

16 272 **DISCUSSION**

18
19 273 In this study we investigated the mechanism of action of GBF1 in HCV infection. Our
20
21 274 results suggest that GBF1 functions in HCV infection by activating class II Arfs. This is
22
23 275 different from what has been reported for in poliovirus, for which its GEF activity and
24
25 276 Arf activation are not required (Belov *et al.*, 2010). A series of GBF1 truncation mutants
26
27 277 inactive for regulating the secretory pathway of the cell, including a construct lacking a
28
29 278 Sec7 domain, were shown to complement BFA inhibition of poliovirus replication. Here
30
31 279 we showed that unlike poliovirus, the same series of GBF1 mutants do not support HCV
32
33 280 replication, clearly indicating a difference of action for GBF1 in poliovirus and HCV
34
35 281 replication. This requirement for GEF activity was confirmed with the use of Arf1
36
37 282 dominant negative mutants.
38
39

41 283 The function of GBF1 in HCV replication is also different from its function in membrane
42
43 284 traffic, as already suggested by our previous study of BFA resistant cell lines (Farhat *et*
44
45 285 *al.*, 2013). Indeed our results suggest that GBF1 may act by activating different pairs of
46
47 286 Arf proteins for the control of HCV replication and of protein secretion. The pair Arf1-
48
49 287 Arf4 mediates GBF1 control of the secretory pathway, as already reported (Volpicelli-
50
51 288 Daley *et al.*, 2005), whereas the pair Arf4-Arf5 mediates GBF1 function in HCV
52
53 289 replication. Recently, Arf4 and Arf5 have also been reported to be involved in dengue
54
55 290 virus infection, although for this virus, the function of this pair of Arfs was suggested to
56
57
58
59
60

1
2
3 291 be involved in the assembly step (Kudelko *et al.*, 2012). Interestingly, the depletion of
4
5 292 Arf4 was reported to protect cells from BFA toxicity, and this effect also depends on
6
7 293 GBF1, Arf1 and Arf5 (Reiling *et al.*, 2013).

8
9
10 294 Based on siRNA experiments, previous studies, including our own, suggested an
11
12 295 involvement of Arf1 in HCV replication (Matto *et al.*, 2011; Zhang *et al.*, 2012; Farhat *et*
13
14 296 *al.*, 2013) that we did not observe in this study. However, in none of these previous
15
16
17 297 studies were the results of siRNA depletions confirmed by re-expressing Arf1, in order
18
19 298 to rule out off-target effects. Off-target effects are always a potential pitfall with siRNA
20
21 299 experiments, as exemplified by our finding that the commercial siRNA pool targeting
22
23 300 Arf1 also inhibits Arf3 expression. Reducing siRNA concentration may help to reduce
24
25 301 off-target effects. Using a more efficient transfection agent allowed us to decrease siRNA
26
27 302 concentration from 80 nM double transfections previously used down to 20 nM single
28
29 303 transfections in this study, with better depletion results on control proteins (data not
30
31 304 shown). With these new experimental conditions, Arf1 depletion resulted in a lower
32
33 305 inhibition of HCV replication (from about 50% in our previous study (Farhat *et al.*,
34
35 306 2013) down to about 30% inhibition in this study) despite efficient reduction of Arf1
36
37 307 mRNA expression. All single Arf depletions similarly yielded low levels of inhibition of
38
39 308 HCV infection, in line with the reported lack of phenotype of single Arf depletion
40
41 309 (Volpicelli-Daley *et al.*, 2005). In contrast, we found a stronger inhibition of HCV
42
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,
46
47 312 because trans dominant phenotypes often result from interactions with regulators (like
48
49 313 Arf-GEFs and Arf-GAPs in this case). This suggests that Arf1, Arf4 and Arf5 share
50
51 314 common regulators, as also indicated by their common inhibition by BFA.
52
53
54
55
56
57
58
59
60

1
2
3 315 Specific phenotypes were already reported for different pairs of Arfs (Volpicelli-Daley *et*
4
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
12
13 320 of Arf1 and Arf4 also displayed a scattered Golgi very similar to what is observed in
14
15 321 BFA-treated cells or in cells depleted of GBF1, whereas cells depleted of Arf4 and Arf5
16
17 322 had a more compact Golgi morphology than control cells. This effect is not mimicked by
18
19 323 GBF1 inhibition, but is rather reminiscent of cells treated with latrunculin B, an inhibitor
20
21 324 of actin polymerization (Valderrama *et al.*, 2001; Dippold *et al.*, 2009). Therefore, the
22
23 325 Arf4-Arf5 pair could potentially activate an effector involved in the regulation of the
24
25 326 actin cytoskeleton. Alternatively, we can also speculate that this effect could result from
26
27 327 an alteration of the metabolism of phosphoinositides, because actin fibers are linked to
28
29 328 Golgi membranes by PI4P-interacting protein GOLPH3 (Dippold *et al.*, 2009). Taken
30
31 329 together these results suggest that GBF1 fulfills its different cellular functions by
32
33 330 activating different pairs of Arfs, which in turn control different aspects of the cellular
34
35 331 metabolism.

36
37 332 An intriguing question is how each pair of Arfs controls specific pathways. This appears
38
39 333 to be a common feature of G-proteins of the Arf family (Panic *et al.*, 2003; Setty *et al.*,
40
41 334 2003; Volpicelli-Daley *et al.*, 2005; Cohen *et al.*, 2007). A redundancy of action is the
42
43 335 simplest explanation, although it is quite difficult to match this model with the diversity
44
45 336 of phenotypes observed with the depletion of different pairs of BFA-sensitive Arfs
46
47 337 (Volpicelli-Daley *et al.*, 2005). Another model for Arf pair specificity could be the
48
49 338 formation of Arf dimers, at the site of interaction with effectors. However, such a kind of
50
51 339 interactions has not yet been reported for any Arf effector to our knowledge. It is also
52
53
54
55
56
57
58
59
60

1
2
3 340 possible that the recruitment or the activation of one Arf would depend on the activation
4
5 341 of the other one. Different models compatible with this possibility have been proposed
6
7 342 (Cohen *et al.*, 2007; Chun *et al.*, 2008). A cascade of recruitment has been reported for
8
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
12
13 345 phenotypes were observed in cells depleted of either protein and in double depleted
14
15 346 cells. However, if the selectivity of the system were not stringent, then it could be
16
17 347 compatible with an inhibition only visible in double depleted cells. It would be
18
19 348 interesting to determine if such a mode of action actually occurs for Arf1-Arf4 and Arf4-
20
21 349 Arf5 pairs.
22
23
24
25

26 350 In conclusion, our results indicate that the role of GBF1 in HCV replication is mediated
27
28 351 by its ArfGEF activity and is potentially related to a function of regulation of lipid
29
30 352 metabolism, rather than of regulation of the protein secretory pathway. Interestingly,
31
32 353 two members of the Arf family, class II Arf4 and Arf5, appear to be of special importance
33
34 354 for mediating GBF1 function in HCV replication. Although very little is known about the
35
36 355 specific functions of class II Arfs, data from our study and from another group
37
38 356 (Takashima *et al.*, 2011) suggest their involvement in the control of lipid metabolism, as
39
40 357 evidenced by the abnormal morphology of lipid droplets in cells depleted of both Arf4
41
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
45
46 360 metabolism to oleic acid-induced lipid droplets was recently described (Wilfling *et al.*,
47
48 361 2014). It is not yet clear how this might relate to our results with class II Arfs-depleted
49
50 362 cells, because the depletion of Arf1 reduced the size of lipid droplets in drosophila cells,
51
52 363 instead of increasing it as we observed for simultaneous depletion of both Arf4 and Arf5
53
54 364 in Huh-7 cells. Further studies will be needed to determine the nature of the enzymes or
55
56
57
58
59
60

1
2
3 365 transporters involved in lipid metabolism that are effectors of Arf4 and Arf5. These
4
5 366 proteins may constitute host factors critical for the replication of HCV and potentially
6
7 367 other RNA viruses.
8

9
10 368

11 369 **EXPERIMENTAL PROCEDURES**

12
13
14 370 **Reagents.** Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline
15
16 371 (PBS), goat and fetal calf sera (FCS), BODIBY 493/503 and 4',6-diamidino-2-
17
18 372 phenylindole (DAPI) were purchased from Life Technologies. Mowiol 4-88 was from
19
20 373 Calbiochem. Protease inhibitors mix (Complete) was from Roche. Other chemicals were
21
22 374 from Sigma.

23
24
25
26 375 **Antibodies.** Mouse anti-E1 mAb A4 (Dubuisson *et al.*, 1994) was produced in vitro by
27
28 376 using a MiniPerm apparatus (Heraeus) as recommended by the manufacturer. Mouse
29
30 377 anti-NS5A mAb 9E10 (Lindenbach *et al.*, 2005) was kindly provided by C. M. Rice (The
31
32 378 Rockefeller University). Mouse anti-NS3 mAb 1848 was from Virostat. Rabbit anti-
33
34 379 human Arf4 mAb (ab171746) was from Abcam. Mouse anti-Arf5 mAb 1B4 was from
35
36 380 Abnova. Sheep anti-TGN46 was from Serotec. Mouse anti-GBF1 and anti-GM130 mAbs
37
38 381 were from Transduction Laboratories. Mouse anti- β -tubulin mAb (TUB 2.1) was from
39
40 382 Sigma. Mouse anti-HSA (ZMHSA1) was from Invitrogen. Goat anti-HSA (507313) was
41
42 383 from Calbiochem. Mouse anti-GFP mAb was from Roche. Alexa 555-conjugated donkey
43
44 384 anti-sheep IgG antibody was from Life Technologies. Peroxidase-conjugated goat anti-
45
46 385 mouse, and anti-sheep IgG, and cyanine 3-conjugated goat anti-mouse IgG were from
47
48 386 Jackson Immunoresearch.

49
50
51
52
53 387 **DNA constructs.** GBF1 deletion mutants were described previously (Niu *et al.*, 2005;
54
55 388 Belov *et al.*, 2010). WT Arf1, Q71L and T31N mutants were kindly provided by B.
56
57 389 Hoflack (Technische Universität Dresden, Germany). Arf coding sequence was excised
58
59
60

1
2
3 390 from pGEM2 plasmids using *Hind*III and *Bam*HI and subcloned in pEGFP-N1 (Clonetechn)
4
5 391 between the same sites to generate Arf1-GFP constructs. To generate Arf1-mCherry
6
7 392 constructs, the GFP coding sequence was excised with AgeI and NotI and replaced by
8
9
10 393 mCherry coding sequence PCR-amplified using primers
11
12 394 GATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAG and
13
14 395 AGAGTCGCGGCCGCTCTACTTGTACAGCTCGTCCATG. Arf4-GFP and Arf5-GFP constructs
15
16 396 were as previously described (Chun *et al.*, 2008) and were obtained through Addgene.
17
18 397 To generate expression vectors for mouse Arf4 and Arf5 used in rescue experiments, the
19
20 398 coding sequences were PCR-amplified from mouse 11-day embryo Marathon-Ready
21
22 399 cDNA (Clonetechn) using primers CTTAAGCTTCCGCCATGGGCCTCACCATC and
23
24
25 400 GTAGGATCCTTAACGTTTTGAAAGTTCATTTGAC (Arf4) or
26
27
28 401 CTTAAGCTTCCGCCATGGGCCTCACGGTG and GTAGGATCCCTAGCGCTTTGACAGCTCGT
29
30 402 (Arf5), and inserted in pCEP4 between *Hind*III and *Bam*HI sites. All constructs were
31
32 403 verified by sequencing.

33
34
35 404 **Cell culture.** Huh-7 (Nakabayashi *et al.*, 1982) cells were grown in Dulbecco's modified
36
37 405 Eagle's medium (DMEM), high glucose modification, supplemented with glutamax-I and
38
39 406 10% FCS.

40
41
42 407 **HCVcc.** The virus JFH1-CSN6A4 used in this study was based on JFH1, and contained cell
43
44 408 culture adaptive mutations (Delgrange *et al.*, 2007) and a reconstituted A4 epitope in E1,
45
46 409 as previously described (Goueslain *et al.*, 2010). The plasmid pJFH1-CSN6A4 was
47
48 410 linearized with *Xba*I and treated with the Mung Bean Nuclease (New England Biolabs).
49
50 411 In vitro transcripts were generated using the Megascript kit according to the
51
52 412 manufacturer's protocol (Ambion). Ten micrograms of in vitro transcribed RNA were
53
54 413 delivered into Huh-7 cells by electroporation as described (Kato *et al.*, 2003). For virus
55
56 414 production, electroporated cells were passaged 3 days after electroporation and grown
57
58
59
60

1
2
3 415 to confluence. The culture medium was collected every day, titrated, aliquoted and
4
5 416 stored at -80°C. For infection assays, sub-confluent naïve Huh-7 cells grown in a P24
6
7 417 well were incubated with 50 µl of this virus preparation diluted to 200 µl of medium for
8
9 418 2 hours (M.O.I. ~0.5), and the inoculate was replaced with fresh culture medium. In
10
11 419 order to reach near 100% infected cells in experiments with Arf1 mutants, cells were
12
13 420 infected with 200 µl of undiluted HCVcc stock with a higher titre, (M.O.I. ~5). In all
14
15 421 experiments, infections were scored at 30 hpi.
16
17

18
19 422 **Adenovirus.** A recombinant defective adenovirus expressing a green fluorescent
20
21 423 protein (EGFP) was as previously described (Farhat *et al.*, 2013). Cells were infected for
22
23 424 1 hour at 37°C, and fixed for 20 minutes with PFA 3% at 16 hours post-infection.
24
25

26 425 **DNA transfection.** Twenty-four hours before transfection, cells were seeded in 24-well
27
28 426 clusters to reach ~70% confluence the next day. Cells were transfected with 0.5 µg of
29
30 427 plasmid DNA mixed with Trans-IT LT1 reagent following the instructions of the
31
32 428 manufacturer (Mirus). Cells transfected with pCEP4-based constructs were co-
33
34 429 transfected with pPUR (clontech) at a 1:20 ratio, and selected for with 5 µg/ml
35
36 430 puromycin for 3 days, and cultured with no puromycin for 4 days before siRNA
37
38 431 transfection.
39
40
41

42
43 432 **RNA interference.** RNA interference experiments were carried out with pools of four
44
45 433 different synthetic double-stranded siRNAs to the same target (on-target plus smart
46
47 434 pool reagents from Dharmacon). Due to an off-target effect with the pool against Arf1 we
48
49 435 used a mix of 2 individual siRNAs (J-011580-05-0005 & J-011580-08-0005). The control
50
51 436 used in this study was the on-target plus non-targeting siRNA #1 (D-001810-01-20).
52
53

54 437 For siRNA transfection, 3 µl of lipofectamine RNAi MAX (Life Technologies) were added
55
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
57
58 439 were spotted in the center of a well. In case of double siRNA transfection, 1.25 µl of each
59
60

1
2
3 440 siRNA was used. Then, the diluted transfection reagent was added to the siRNA and the
4
5 441 mixture was incubated for 30 min at room temperature. At the end of this incubation,
6
7 442 2.5×10^5 freshly trypsinized cells in a volume of 2 ml of culture medium were added to the
8
9 443 transfection mix and the cells were returned to 37°C. Cells were trypsinized 24 h later
10
11 444 and seeded on glass cover slips, and analyzed by immunofluorescence 3 or 4 days after
12
13 445 siRNA transfection. For quantifying HCV infection, siRNA-treated cells were infected 48
14
15 446 h after trypsinization. Just before infection, extra wells of cells treated with each siRNA
16
17 447 were used to extract RNA for quantifying the depletion efficiency. Infected cells were
18
19 448 stained with anti-E1 mAb A4 and DAPI at 30 hpi and HCV-infected were counted. At
20
21 449 least 5000 cells were counted per experiment for each condition.
22
23
24

25
26 450 **Immunoblotting.** Cells were rinsed 3 times with cold PBS, and lysed at 4°C for 20 min
27
28 451 in a buffer containing 50 mM TrisCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1% Triton- X,
29
30 452 0.1% SDS, 1 mM PMSF, and a mix of protease inhibitors (Complete). Insoluble material
31
32 453 was removed by centrifugation at 4°C. The protein content was determined by the
33
34 454 bicinchoninic acid method as recommended by the manufacturer (Sigma), using bovine
35
36 455 serum albumin as the standard. The proteins were then resolved by SDS-PAGE and
37
38 456 transferred onto nitrocellulose membranes (Hybond-ECL; Amersham) using a Trans-
39
40 457 Blot apparatus (Bio-Rad). Proteins of interest were revealed with specific primary
41
42 458 antibodies, followed by species-specific secondary antibodies conjugated to peroxidase.
43
44 459 Proteins were visualized using enhanced chemiluminescence (ECL Plus, GE healthcare).
45
46 460 The signals were recorded using a LAS 3000 apparatus (Fujifilm). Quantification of
47
48 461 unsaturated signals was carried out using the gel quantification function of ImageJ.
49
50
51

52
53 462 **Immunofluorescence microscopy.** Indirect immunofluorescence labeling was
54
55 463 performed as previously described (Rouillé *et al.*, 2006). Lipid droplets were stained
56
57 464 with BODIBY 493/503 (0.5 µg/ml; Invitrogen) for 10 min at room temperature. Nuclei
58
59
60

1
2
3 465 were stained with DAPI. For colocalization experiments, confocal microscopy was
4
5 466 carried out with an LSM780 confocal microscope (Zeiss) using a 63X oil immersion
6
7 467 objective with a 1.4 numerical aperture. Signals were sequentially collected using single
8
9 468 fluorescence excitation and acquisition settings to avoid crossover. Images were
10
11 469 processed using Adobe Photoshop software CS4.

12
13
14 470 ***BFA rescue experiments.*** Cells were transfected with GBF1 mutants and then infected
15
16 471 with JFH1-CSN6A4 48 h post transfection in the presence of 50 ng/ml of BFA, or the
17
18 472 corresponding volume of ethanol (BFA solvent). Cells were lysed at 30 hpi and the
19
20 473 expression levels of HCV E1 (A4) were measured by Western blot. The expression level
21
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.

25
26
27
28 476 ***Secretion assays.*** Sub-confluent cell cultures grown in 12-well plates were incubated
29
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
33
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).
37
38 481 Apolipoprotein E was quantified using a commercial ELISA kit from Mabtech. The
39
40 482 percentage of secretion was calculated as the percentage of HSA/apoE in the medium
41
42 483 divided by the total amount of HSA/apoE in the medium and the lysate.

43
44
45
46
47 484 ***Replication assay.*** The construct used for the replication assay (HCVcc-Rluc/ Δ E1E2)
48
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
52
53 487 luciferase activity was measured 4 h, 24 h, 48 h and 72 h post-electroporation using the
54
55 488 *Renilla* luciferase assay system kit from Promega.

1
2
3 489 **Quantitative RT-PCR.** Total RNA was extracted from siRNA-transfected and control
4
5 490 cells using Nucleospin RNA II extraction kit (Macherey-Nagel), which includes a DNase I
6
7 491 treatment. cDNA was obtained from RNA using the High Capacity cDNA Reverse
8
9 492 transcription kit (Life Technologies) in a final volume of 20 μ l. Quantitative RT-PCR
10
11 493 analysis was performed using the Taqman® pre-designed gene expression assay
12
13 494 approach (Applied Biosystems), using 1 μ l of cDNA and premade probes designed by the
14
15 495 manufacturer. The ratio of the mRNA level of each gene to that of large ribosomal
16
17 496 protein P0 (RPLP0) endogenous control mRNA was calculated by the $\Delta\Delta$ Ct method
18
19 497 (Livak and Schmittgen, 2001), and a value of 100 was assigned to control siRNA-
20
21 498 transfected cells. Each experiment was performed in triplicate and repeated three times.
22
23
24
25
26 499 **HCVpp.** The luciferase-based HCV-pseudotyped retroviral particles (HCVpp) infection
27
28 500 assay was performed as previously described (Op De Beeck *et al.*, 2004).
29
30

31 501

32 502 **ACKNOWLEDGMENTS**

33
34
35 503 This work was supported by grants from the “Agence Nationale de la Recherche sur le
36
37 504 SIDA et les hépatites virales” (ANRS) and “Agence Nationale de la Recherche” (ANR)
38
39 505 through ERA-NET Infect-ERA program (ANR-13-IFEC-0002-01).
40
41

42 506 We thank B. Hoflack, C.M. Rice and T. Wakita for providing us with reagents. We are
43
44 507 grateful to Sophana Ung for his assistance in the illustrations. Some data were generated
45
46 508 with the help of the Imaging Core Facility of the campus Calmette (BICeL).
47
48

49 509 The authors have no conflict of interest to declare.
50
51
52
53
54
55
56
57
58
59
60

1
2
3 510 REFERENCES
45 511
6

7 512 Afzal, M.S., Alsaleh, K., Farhat, R., Belouzard, S., Danneels, A., Descamps, V., *et al.* (2014)
8 513 Regulation of core expression during the hepatitis C virus life cycle. *Journal of General*
9 514 *Virology* **96**:311-321.

11 515 Beller, M., Sztalryd, C., Southall, N., Bell, M., Jäckle, H., Auld, D.S., and Oliver, B. (2008)
12 516 COPI complex is a regulator of lipid homeostasis. *PLoS Biol* **6**: e292.

14 517 Belov, G.A., Feng, Q., Nikovics, K., Jackson, C.L., and Ehrenfeld, E. (2008) A critical role of
15 518 a cellular membrane traffic protein in poliovirus RNA replication. *PLoS Pathog* **4**:
16 519 e1000216.

18 520 Belov, G.A., Kovtunovych, G., Jackson, C.L., and Ehrenfeld, E. (2010) Poliovirus replication
19 521 requires the N-terminus but not the catalytic Sec7 domain of ArfGEF GBF1. *Cell Microbiol*
20 522 **12**: 1463–1479.

22 523 Belov, G.A., Nair, V., Hansen, B.T., Hoyt, F.H., Fischer, E.R., and Ehrenfeld, E. (2012)
23 524 Complex dynamic development of poliovirus membranous replication complexes. *J Virol*
24 525 **86**: 302–312.

26 526 Berger, K.L., Cooper, J.D., Heaton, N.S., Yoon, R., Oakland, T.E., Jordan, T.X., *et al.* (2009)
27 527 Roles for endocytic trafficking and phosphatidylinositol 4-kinase III alpha in hepatitis C
28 528 virus replication. *Proc Natl Acad Sci USA* **106**: 7577–7582.

30 529 Borawski, J., Troke, P., Puyang, X., Gibaja, V., Zhao, S., Mickanin, C., *et al.* (2009) Class III
31 530 phosphatidylinositol 4-kinase alpha and beta are novel host factor regulators of
32 531 hepatitis C virus replication. *J Virol* **83**: 10058–10074.

34 532 Bouvet, S., Golinelli-Cohen, M.-P., Contremoulins, V., and Jackson, C.L. (2013) Targeting
35 533 of the Arf-GEF GBF1 to lipid droplets and Golgi membranes. *J Cell Sci* **126**: 4794–4805.

37 534 Bui, Q.T., Golinelli-Cohen, M.-P., and Jackson, C.L. (2009) Large Arf1 guanine nucleotide
38 535 exchange factors: evolution, domain structure, and roles in membrane trafficking and
39 536 human disease. *Mol Genet Genomics* **282**: 329–350.

41 537 Carpp, L.N., Rogers, R.S., Moritz, R.L., and Aitchison, J.D. (2014) Quantitative proteomic
42 538 analysis of host-virus interactions reveals a role for Golgi brefeldin A resistance factor 1
43 539 (GBF1) in dengue infection. *Mol Cell Proteomics* **13**: 2836–2854.

45 540 Cherry, S., Kunte, A., Wang, H., Coyne, C., Rawson, R.B., and Perrimon, N. (2006) COPI
46 541 activity coupled with fatty acid biosynthesis is required for viral replication. *PLoS*
47 542 *Pathog* **2**: e102.

49 543 Christis, C., and Munro, S. (2012) The small G protein Arl1 directs the trans-Golgi-
50 544 specific targeting of the Arf1 exchange factors BIG1 and BIG2. *J Cell Biol* **196**: 327–335.

52 545 Chun, J., Shapovalova, Z., Dejgaard, S.Y., Presley, J.F., and Melançon, P. (2008)
53 546 Characterization of class I and II ADP-ribosylation factors (Arfs) in live cells: GDP-bound
54 547 class II Arfs associate with the ER-Golgi intermediate compartment independently of

- 1
2
3 548 GBF1. *Mol Biol Cell* **19**: 3488–3500.
- 4
5 549 Claude, A., Zhao, B.P., Kuziemsky, C.E., Dahan, S., Berger, S.J., Yan, J.P., *et al.* (1999) GBF1:
6 550 A novel Golgi-associated BFA-resistant guanine nucleotide exchange factor that displays
7 551 specificity for ADP-ribosylation factor 5. *J Cell Biol* **146**: 71–84.
- 8
9 552 Cohen, L.A., Honda, A., Varnai, P., Brown, F.D., Balla, T., and Donaldson, J.G. (2007) Active
10 553 Arf6 recruits ARNO/cytohesin GEFs to the PM by binding their PH domains. *Mol Biol Cell*
11 554 **18**: 2244–2253.
- 12
13 555 Dascher, C., and Balch, W.E. (1994) Dominant inhibitory mutants of ARF1 block
14 556 endoplasmic reticulum to Golgi transport and trigger disassembly of the Golgi apparatus.
15 557 *J Biol Chem* **269**: 1437–1448.
- 16
17
18 558 Delgrange, D., Pillez, A., Castelain, S., Cocquerel, L., Rouillé, Y., Dubuisson, J., *et al.* (2007)
19 559 Robust production of infectious viral particles in Huh-7 cells by introducing mutations in
20 560 hepatitis C virus structural proteins. *J Gen Virol* **88**: 2495–2503.
- 21
22 561 Dippold, H.C., Ng, M.M., Farber-Katz, S.E., Lee, S.-K., Kerr, M.L., Peterman, M.C., *et al.*
23 562 (2009) GOLPH3 bridges phosphatidylinositol-4- phosphate and actomyosin to stretch
24 563 and shape the Golgi to promote budding. *Cell* **139**: 337–351.
- 25
26
27 564 Donaldson, J.G., and Jackson, C.L. (2011) ARF family G proteins and their regulators:
28 565 roles in membrane transport, development and disease. *Nat Rev Mol Cell Biol* **12**: 362–
29 566 375.
- 30
31 567 Dubuisson, J., Hsu, H.H., Cheung, R.C., Greenberg, H.B., Russell, D.G., and Rice, C.M. (1994)
32 568 Formation and intracellular localization of hepatitis C virus envelope glycoprotein
33 569 complexes expressed by recombinant vaccinia and Sindbis viruses. *J Virol* **68**: 6147–
34 570 6160.
- 35
36
37 571 Egger, D., Wölk, B., Gosert, R., Bianchi, L., Blum, H.E., Moradpour, D., and Bienz, K. (2002)
38 572 Expression of hepatitis C virus proteins induces distinct membrane alterations including
39 573 a candidate viral replication complex. *J Virol* **76**: 5974–5984.
- 40
41 574 Farhat, R., Goueslain, L., Wychowski, C., Belouzard, S., Fénéant, L., Jackson, C.L., *et al.*
42 575 (2013) Hepatitis C virus replication and Golgi function in brefeldin a-resistant
43 576 hepatoma-derived cells. *PLoS ONE* **8**: e74491.
- 44
45 577 Ferraris, P., Blanchard, E., and Roingeard, P. (2010) Ultrastructural and biochemical
46 578 analyses of hepatitis C virus-associated host cell membranes. *Journal of General Virology*
47 579 **91**: 2230–2237.
- 48
49 580 Gazina, E.V., Mackenzie, J.M., Gorrell, R.J., and Anderson, D.A. (2002) Differential
50 581 requirements for COPI coats in formation of replication complexes among three genera
51 582 of Picornaviridae. *J Virol* **76**: 11113–11122.
- 52
53
54 583 Gillespie, L.K., Hoenen, A., Morgan, G., and Mackenzie, J.M. (2010) The endoplasmic
55 584 reticulum provides the membrane platform for biogenesis of the flavivirus replication
56 585 complex. **84**: 10438–10447.
- 57
58 586 Goueslain, L., Alsaleh, K., Horellou, P., Roingeard, P., Descamps, V., Duverlie, G., *et al.*
59
60

- 1
2
3 587 (2010) Identification of GBF1 as a cellular factor required for hepatitis C virus RNA
4 588 replication. *J Virol* **84**: 773–787.
- 5
6 589 Guo, Y., Walther, T.C., Rao, M., Stuurman, N., Goshima, G., Terayama, K., *et al.* (2008)
7 590 Functional genomic screen reveals genes involved in lipid-droplet formation and
8 591 utilization. *Nature* **453**: 657–661.
- 9
10 592 Kato, T., Date, T., Miyamoto, M., Furusaka, A., Tokushige, K., Mizokami, M., and Wakita, T.
11 593 (2003) Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon.
12 594 *Gastroenterology* **125**: 1808–1817.
- 13
14
15 595 Knoops, K., Kikkert, M., Worm, S.H.E.V.D., Zevenhoven-Dobbe, J.C., van der Meer, Y.,
16 596 Koster, A.J., *et al.* (2008) SARS-coronavirus replication is supported by a
17 597 reticulovesicular network of modified endoplasmic reticulum. *PLoS Biol* **6**: e226.
- 18
19 598 Kudelko, M., Brault, J.-B., Kwok, K., Li, M.Y., Pardigon, N., Peiris, J.S.M., *et al.* (2012) Class
20 599 II ADP-ribosylation factors are required for efficient secretion of dengue viruses. *Journal*
21 600 *of Biological Chemistry* **287**: 767–777.
- 22
23
24 601 Lanke, K.H.W., van der Schaar, H.M., Belov, G.A., Feng, Q., Duijsings, D., Jackson, C.L., *et al.*
25 602 (2009) GBF1, a guanine nucleotide exchange factor for Arf, is crucial for coxsackievirus
26 603 B3 RNA replication. *J Virol* **83**: 11940–11949.
- 27
28 604 Limpens, R.W.A.L., van der Schaar, H.M., Kumar, D., Koster, A.J., Snijder, E.J., van
29 605 Kuppeveld, F.J.M., and Bárcena, M. (2011) The transformation of enterovirus replication
30 606 structures: a three-dimensional study of single- and double-membrane compartments.
31 607 *MBio* **2**.
- 32
33 608 Lindenbach, B.D., Evans, M.J., Syder, A.J., Wölk, B., Tellinghuisen, T.L., Liu, C.C., *et al.*
34 609 (2005) Complete replication of hepatitis C virus in cell culture. *Science* **309**: 623–626.
- 35
36 610 Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using
37 611 real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**: 402–408.
- 38
39 612 Matto, M., Sklan, E.H., David, N., Melamed-Book, N., Casanova, J.E., Glenn, J.S., and Aroeti,
40 613 B. (2011) Role for ADP Ribosylation Factor 1 in the Regulation of Hepatitis C Virus
41 614 Replication. *J Virol* **85**: 946–956.
- 42
43
44 615 Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T., and Sato, J. (1982) Growth of human
45 616 hepatoma cells lines with differentiated functions in chemically defined medium. *Cancer*
46 617 *Res* **42**: 3858–3863.
- 47
48 618 Niu, T.-K., Pfeifer, A.C., Lippincott-Schwartz, J., and Jackson, C.L. (2005) Dynamics of
49 619 GBF1, a Brefeldin A-sensitive Arf1 exchange factor at the Golgi. *Mol Biol Cell* **16**: 1213–
50 620 1222.
- 51
52
53 621 Op De Beeck, A., Voisset, C., Bartosch, B., Ciczora, Y., Cocquerel, L., Keck, Z., *et al.* (2004)
54 622 Characterization of functional hepatitis C virus envelope glycoproteins. *J Virol* **78**: 2994–
55 623 3002.
- 56
57 624 Panic, B., Whyte, J.R.C., and Munro, S. (2003) The ARF-like GTPases Arl1p and Arl3p act
58 625 in a pathway that interacts with vesicle-tethering factors at the Golgi apparatus. *Curr*

- 1
2
3 626 *Biol* **13**: 405–410.
4
5 627 Reiling, J.H., Olive, A.J., Sanyal, S., Carette, J.E., Brummelkamp, T.R., Ploegh, H.L., *et al.*
6 628 (2013) A CREB3-ARF4 signalling pathway mediates the response to Golgi stress and
7 629 susceptibility to pathogens. *Nat Cell Biol.*
- 8
9 630 Richards, A.L., Soares-Martins, J.A.P., Riddell, G.T., and Jackson, W.T. (2014) Generation
10 631 of unique poliovirus RNA replication organelles. *MBio* **5**: e00833–13.
- 11
12 632 Romero-Brey, I., Merz, A., Chiramel, A., Lee, J.Y., Chlanda, P., Haselman, U., *et al.* (2012)
13 633 Three-dimensional architecture and biogenesis of membrane structures associated with
14 634 hepatitis C virus replication. *PLoS Pathog* **8**: e1003056.
- 15
16
17 635 Rouillé, Y., Helle, F., Delgrange, D., Roingeard, P., Voisset, C., Blanchard, E., *et al.* (2006)
18 636 Subcellular localization of hepatitis C virus structural proteins in a cell culture system
19 637 that efficiently replicates the virus. *J Virol* **80**: 2832–2841.
- 20
21 638 Schmeiser, S., Mast, J., Thiel, H.-J., and König, M. (2014) Morphogenesis of pestiviruses:
22 639 new insights from ultrastructural studies of strain Giraffe-1. *J Virol* **88**: 2717–2724.
- 23
24 640 Setty, S.R.G., Shin, M.E., Yoshino, A., Marks, M.S., and Burd, C.G. (2003) Golgi recruitment
25 641 of GRIP domain proteins by Arf-like GTPase 1 is regulated by Arf-like GTPase 3. *Curr Biol*
26 642 **13**: 401–404.
- 27
28
29 643 Snooks, M.J., Bhat, P., Mackenzie, J., Counihan, N.A., Vaughan, N., and Anderson, D.A.
30 644 (2008) Vectorial entry and release of hepatitis A virus in polarized human hepatocytes. *J*
31 645 *Virol* **82**: 8733–8742.
- 32
33 646 Soni, K.G., Mardones, G.A., Sougrat, R., Smirnova, E., Jackson, C.L., and Bonifacino, J.S.
34 647 (2009) Coatamer-dependent protein delivery to lipid droplets. *J Cell Sci* **122**: 1834–1841.
- 35
36 648 Szul, T., Grabski, R., Lyons, S., Morohashi, Y., Shestopal, S., Lowe, M., and Sztul, E. (2007)
37 649 Dissecting the role of the ARF guanine nucleotide exchange factor GBF1 in Golgi
38 650 biogenesis and protein trafficking. *J Cell Sci* **120**: 3929–3940.
- 39
40
41 651 Tai, A.W., Benita, Y., Peng, L.F., Kim, S.-S., Sakamoto, N., Xavier, R.J., and Chung, R.T.
42 652 (2009) A Functional Genomic Screen Identifies Cellular Cofactors of Hepatitis C Virus
43 653 Replication. *Cell Host Microbe* **5**: 298–307.
- 44
45 654 Takashima, K., Saitoh, A., Hirose, S., Nakai, W., Kondo, Y., Takasu, Y., *et al.* (2011) GBF1-
46 655 Arf-COPI-ArfGAP-mediated Golgi-to-ER transport involved in regulation of lipid
47 656 homeostasis. *Cell Struct Funct* **36**: 223–235.
- 48
49
50 657 Trotard, M., Lepère-Douard, C., Régeard, M., Piquet-Pellorce, C., Lavillette, D., Cosset, F.-L.,
51 658 *et al.* (2009) Kinases required in hepatitis C virus entry and replication highlighted by
52 659 small interference RNA screening. *The FASEB Journal* **23**: 3780–3789.
- 53
54 660 Ulasli, M., Verheije, M.H., de Haan, C.A.M., and Reggiori, F. (2010) Qualitative and
55 661 quantitative ultrastructural analysis of the membrane rearrangements induced by
56 662 coronavirus. *Cell Microbiol* **12**: 844–861.
- 57
58
59 663 Vaillancourt, F.H., Pilote, L., Cartier, M., Lippens, J., Liuzzi, M., Bethell, R.C., *et al.* (2009)

- 1
2
3 664 Identification of a lipid kinase as a host factor involved in hepatitis C virus RNA
4 665 replication. *Virology* **387**: 5–10.
- 5
6 666 Valderrama, F., Durán, J.M., Babià, T., Barth, H., Renau-Piqueras, J., and Egea, G. (2001)
7 667 Actin microfilaments facilitate the retrograde transport from the Golgi complex to the
8 668 endoplasmic reticulum in mammalian cells. *Traffic* **2**: 717–726.
- 9
10 669 Verheije, M.H., Raaben, M., Mari, M., Lintelo, te, E.G., Reggiori, F., van Kuppeveld, F.J.M., *et*
11 670 *al.* (2008) Mouse hepatitis coronavirus RNA replication depends on GBF1-mediated
12 671 ARF1 activation. *PLoS Pathog* **4**: e1000088.
- 13
14
15 672 Volpicelli-Daley, L.A., Li, Y., Zhang, C.-J., and Kahn, R.A. (2005) Isoform-selective effects of
16 673 the depletion of ADP-ribosylation factors 1-5 on membrane traffic. *Mol Biol Cell* **16**:
17 674 4495–4508.
- 18
19 675 Wang, J., Du, J., and Jin, Q. (2014) Class I ADP-Ribosylation Factors Are Involved in
20 676 Enterovirus 71 Replication. *PLoS ONE* **9**: e99768.
- 21
22 677 Wang, J., Wu, Z., and Jin, Q. (2012) COPI is required for enterovirus 71 replication. *PLoS*
23 678 *ONE* **7**: e38035.
- 24
25
26 679 Welsch, S., Miller, S., Romero-Brey, I., Merz, A., Bleck, C.K.E., Walther, P., *et al.* (2009)
27 680 Composition and three-dimensional architecture of the dengue virus replication and
28 681 assembly sites. *Cell Host Microbe* **5**: 365–375.
- 29
30 682 Wilfling, F., Thiam, A.R., Olarte, M.J., Wang, J., Beck, R., Gould, T.J., *et al.* (2014) Arf1/COPI
31 683 machinery acts directly on lipid droplets and enables their connection to the ER for
32 684 protein targeting. *Elife* **3**: e01607–e01607.
- 33
34 685 Wright, J., Kahn, R.A., and Sztul, E. (2014) Regulating the large Sec7 ARF guanine
35 686 nucleotide exchange factors: the when, where and how of activation. *Cell Mol Life Sci* **71**:
36 687 3419–3438.
- 37
38
39 688 Zhang, L., Hong, Z., Lin, W., Shao, R.-X., Goto, K., Hsu, V.W., and Chung, R.T. (2012) ARF1
40 689 and GBF1 Generate a PI4P-Enriched Environment Supportive of Hepatitis C Virus
41 690 Replication. *PLoS ONE* **7**: e32135.
- 42
43 691
44
45 692
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 693 **FIGURES LEGENDS**
4
5 694
6
7 695

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.
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38

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
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 718 infected with HCV and the infection was quantified in at least 100 fluorescent cells per
4
5 719 experiment for each construct (black series). For mock-transfected cells, the infection in
6
7 720 the total population was quantified. A similar analysis was performed with an
8
9 721 adenovirus expressing GFP in cells expressing the mCherry constructs (grey series). The
10
11 722 results are averages of 3 independent experiments (\pm SD), * $P < 0.05$, *** $P < 0.001$.
12
13
14 723

15
16 724 **Figure 3. Impact of Arf proteins depletion on HCV replication.** (A) Huh-7 cells were
17
18 725 transfected with indicated siRNA, infected with HCV or adenovirus at 72 h post
19
20 726 transfection, fixed at 30 hpi and processed for detection of infected cells by
21
22 727 immunofluorescence. Infection of non-targeting siRNA-treated samples is expressed as
23
24 728 100% (*** $P < 0.001$, 1-way ANOVA). (B) Total RNA was extracted from siRNA-
25
26 729 transfected cells at 72 h post transfection, and the indicated mRNAs were quantified by
27
28 730 RT-qPCR. mRNA amounts in non-targeting siRNA-treated samples are expressed as
29
30 731 100%. (C) pCEP4, pCEP4-Arf4m or pCEP4-Arf5m (both from mouse) transfected Huh-7
31
32 732 cells were transfected with indicated siRNA and analyzed by immunoblotting at 3 days
33
34 733 post transfection using Arf4, Arf5 and tubulin antibodies. Note that the anti-Arf4
35
36 734 antibody does not detect the transfected murine Arf4. (D) pCEP4, pCEP4-Arf4m or
37
38 735 pCEP4-Arf5m-transfected Huh-7 cells were transfected with indicated siRNA and
39
40 736 infected with HCV 3 days later. Cells were fixed at 30 hpi, labeled with an anti-E1
41
42 737 antibody and the number of infected cells was counted. The number of infected cells in
43
44 738 pCEP4/non-targeting siRNA-transfected cells was expressed as 100%. Error bars
45
46 739 represent standard error of the means (SEM) from 4 independent experiments (**
47
48 740 $P < 0.01$, *** $P < 0.001$, pCEP4-Arf vs pCEP4, 2-way ANOVA). (E) Huh-7 cells were
49
50 741 transfected with indicated siRNAs and electroporated 3 days post transfection with a
51
52 742 recombinant HCV genome containing a deletion in E1E2 and expressing *Renilla*
53
54
55
56
57
58
59
60

1
2
3 743 luciferase. Samples were harvested for luciferase assay at 4, 24, 48, and 72 h post
4
5 744 electroporation. Error bars indicate standard errors of the mean for 3 independent
6
7 745 experiments performed in triplicate.
8

9
10 746

11 747 **Figure 4. Impact of Arf pair depletion on the secretory pathway.** (A) Huh-7 cells
12
13
14 748 transfected with the indicated siRNA were seeded in 12-well plates, and cultured for 24
15
16 749 h. The amounts of human serum albumin (HSA) and of apolipoprotein E (apoE) in the
17
18 750 conditioned culture media and in cell lysates were quantified with an ELISA assay and
19
20 751 expressed as percentage of secretion. Error bars represent standard deviation of 3
21
22 752 independent experiments performed in duplicate (***) $P < 0.001$, 1-way ANOVA). (B) Huh-
23
24
25 753 7 were transfected with indicated siRNAs and fixed 72 h later. Cells were fixed and
26
27 754 processed for immunofluorescent detection of ERGIC53, GBF1, GM130 and TGN46
28
29 755 (white). Nuclei were stained with DAPI (blue). Representative confocal images are
30
31 756 shown. Bar, 20 μm .
32
33
34
35
36

37 758 **Figure 5. Impact of Arf4 and Arf5 depletion on lipid droplets.** (A) Huh-7 cells were
38
39 759 transfected with indicated siRNAs and fixed 72 h later. Cells were fixed and processed
40
41 760 for immunofluorescent detection of GM130 (red). Lipid droplets were stained with
42
43 761 BODIBY 493/503 (green) and nuclei with DAPI (blue). (B) Huh-7 cells were cultured for
44
45 762 24 h in the presence or the absence of BFA (50 ng/ml), and processed for the detection
46
47 763 of lipid droplets and nuclei. Representative confocal images are shown. Bars, 20 μm .
48
49
50
51

52
53 764

54 765 **LEGENDS TO THE SUPPLEMENTARY FIGURES**

55 766
56
57
58
59
60

1
2
3 767 **Figure S1. Off-target effect of Arf1 siRNA pool.** Huh-7 cells were transfected with
4
5 768 indicated siRNA and total RNA was extracted 24 h post transfection. Arf1 and Arf3
6
7 769 mRNAs were quantified by RT-qPCR and mRNA amounts in non-targeting siRNA-treated
8
9 770 samples are expressed as 100%.

10
11 771

12
13 772 **Figure S2. Impact of class II Arf proteins depletion on HCVpp, VSV-Gpp and**
14
15
16 773 **RD114pp entry.** Huh-7 cells were transfected with indicated siRNA and infected with
17
18 774 HCVpp, VSV-Gpp or RD114pp 3 days post transfection. Samples were harvested for
19
20 775 luciferase assay at 48 hpi. Error bars indicate standard deviation for 3 independent
21
22 776 experiments performed in triplicate.

23
24
25 777

26
27
28 778 **Figure S3. Immunofluorescence analysis of Arf4 and Arf5 intracellular localization**
29
30 779 **in non infected Huh-7 cells.** Huh-7 cells transfected with Arf4-GFP or Arf5-GFP
31
32 780 expression plasmids were processed for immunofluorescent detection of GM130 (A) or
33
34 781 GBF1 (B). Representative confocal images of transfected cells are shown together with
35
36 782 the merge image. Bars, 20 μ m.

37
38
39 783

40
41 784 **Figure S4. Immunofluorescence analysis of Arf4 and Arf5 intracellular localization**
42
43 785 **in infected Huh-7 cells.** Huh-7 cells infected with HCV and transfected with Arf4-GFP or
44
45 786 Arf5-GFP expression plasmids were processed for immunofluorescent detection of NS3
46
47 787 (A) or NS5A (B). Representative confocal images of transfected cells are shown together
48
49 788 with the merge image. Bars, 20 μ m.

50
51
52 789

53
54
55 790 **Figure S5. Impact of the depletion of different Arf pairs on lipid droplets.** (A) Huh-7
56
57 791 were transfected with indicated siRNAs and fixed 72 h later. Cells were fixed and

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

For Peer Review

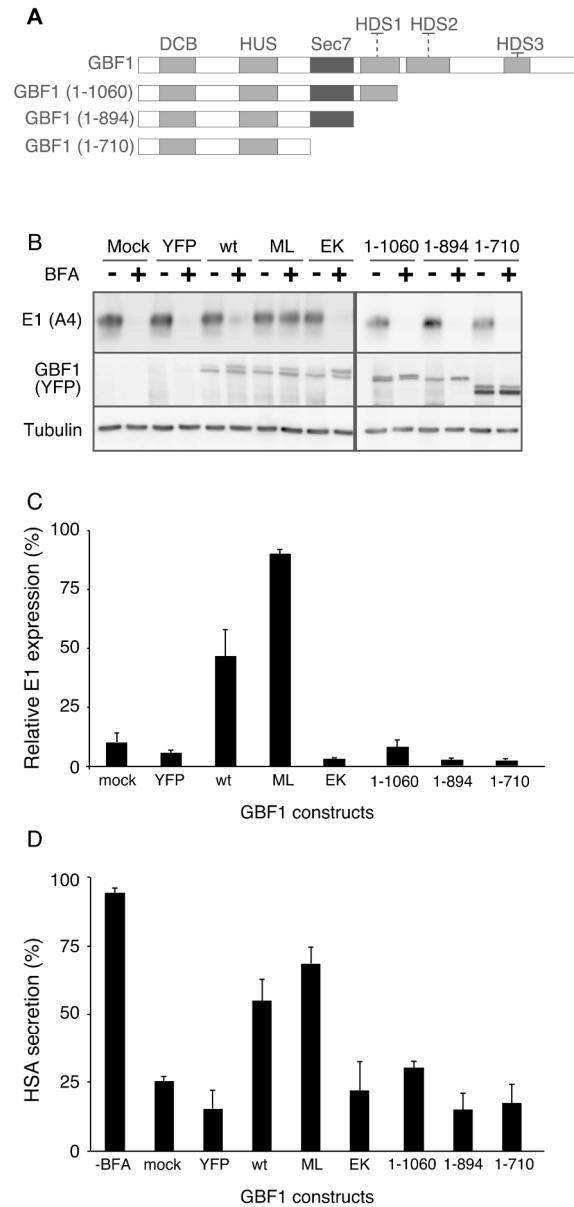


Figure 1. Activity of GBF1 truncation mutants in HCV replication. (A) Schematic representation of GBF1 deletion constructs. Conserved domains are indicated in grey and black. (B) Huh-7 cells were transfected with the indicated constructs, infected 16 h later in the presence or the absence of BFA (50 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.

169x356mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

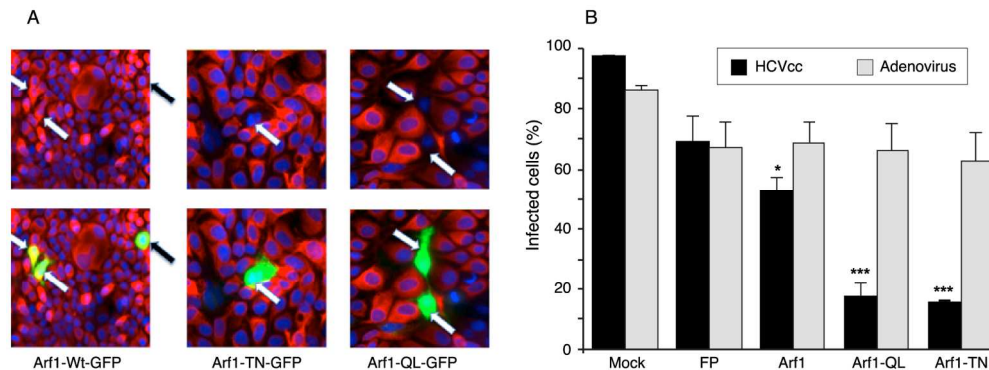


Figure 2. Effect of T31N and Q71L of Arf1 mutants on HCV infection. (A) Huh-7 cells were transfected with plasmids expressing Arf1-GFP (WT), Arf1 T31N-GFP or Arf1 Q71L-GFP, and infected with HCV 16 hours after transfection. The cells were fixed at 30 hpi and infected cells were labeled with an anti-E1 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), * $P < 0.05$, *** $P < 0.001$.

166x61mm (300 x 300 DPI)

Review

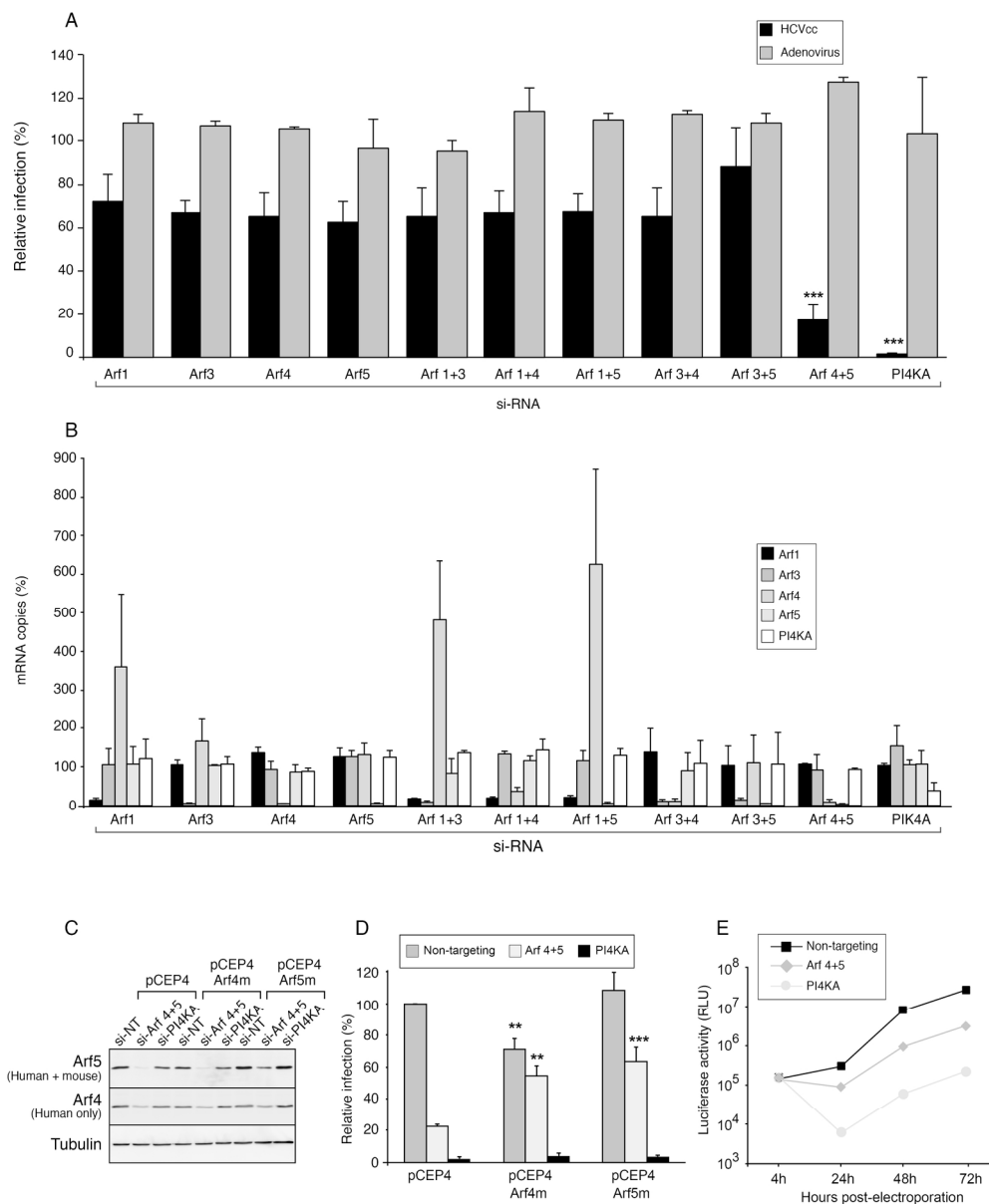


Figure 3. Impact of Arf proteins depletion on HCV replication. (A) Huh-7 cells were transfected with indicated siRNA, infected with HCV or adenovirus at 72 h post transfection, fixed at 30 hpi and processed for detection of infected cells by immunofluorescence. Infection of non-targeting siRNA-treated 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

1
2
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.
7 192x231mm (300 x 300 DPI)
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

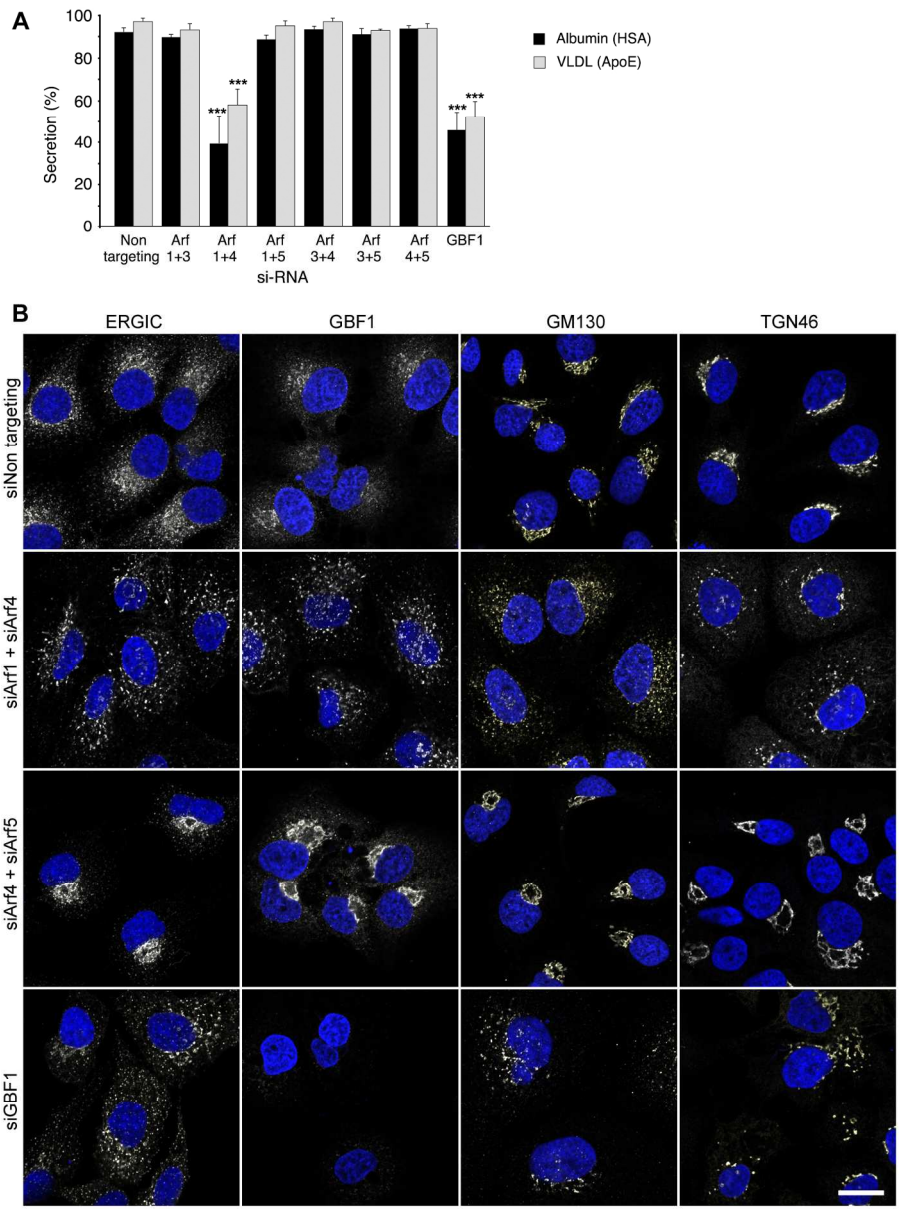


Figure 4. Impact of Arf pair depletion on the secretory pathway. (A) Huh-7 cells transfected with the indicated siRNA were seeded in 12-well plates, and cultured for 24 h. The amounts of human serum albumin (HSA) and of apolipoprotein E (apoE) in the conditioned culture media and in cell lysates were 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 μ m.

157x211mm (300 x 300 DPI)

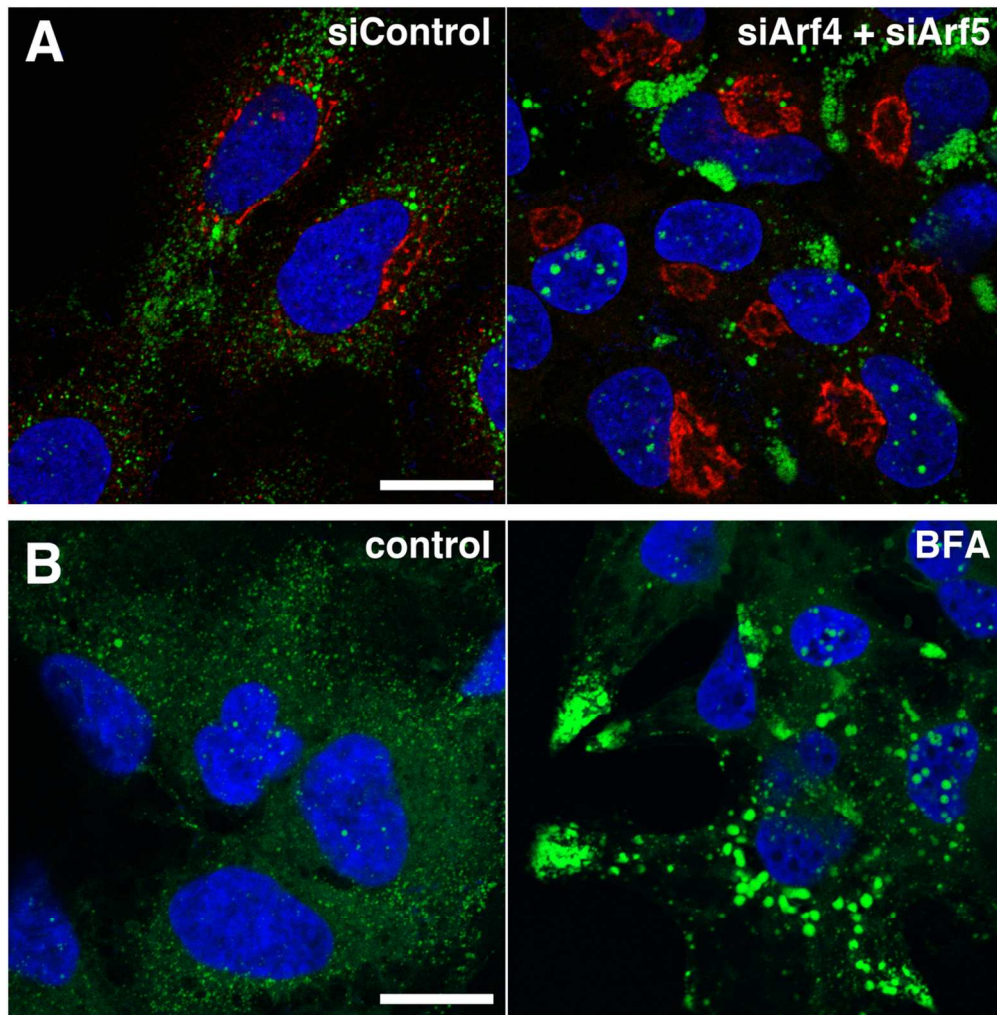


Figure 5. Impact of Arf4 and Arf5 depletion on lipid droplets. (A) Huh-7 cells were transfected with indicated siRNAs and fixed 72 h later. Cells were fixed and processed for immunofluorescent detection of GM130 (red). Lipid droplets were stained with BODIBY 493/503 (green) and nuclei with DAPI (blue). (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)