1   Rab1b-GBF1-ARF1 secretory pathway axis is required for Birnavirus replication.

2   Running title: Rab1b-GBF1-ARF1 in Birnavirus replication

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26

27   **ABSTRACT**

28

29   Birnaviruses are members of the *Birnaviridae* family, responsible for major economic

30   losses to poultry and aquaculture. The family is composed of non-enveloped viruses with a

31   segmented double-stranded RNA (dsRNA) genome. Infectious bursal disease virus

32   (IBDV), the prototypic family member, is the etiological agent of Gumboro disease, a

33   highly contagious immunosuppressive disease in the poultry industry worldwide. We

34   previously demonstrated that IBDV hijacks the endocytic pathway for establishing the viral

35   replication complexes on endosomes associated with the Golgi complex (GC). In this work,

36   we report that IBDV reorganizes the GC to localize the endosome-associated replication

37   complexes without affecting its secretory functionality. Analyzing crucial proteins involved

38   in the secretory pathway, we showed the essential requirement of Rab1b for viral

39   replication. Rab1b comprises a key regulator of GC transport and we demonstrate that

40   transfecting the negative mutant Rab1b N121I or knocking down Rab1b expression by

41   RNA interference significantly reduces the yield of infectious viral progeny. Furthermore,

42   we showed that the Rab1b downstream effector Golgi-specific BFA resistance factor 1

43   (GBF1), which activates the small GTPase ADP-ribosylation factor 1 (ARF1), is required

44   for IBDV replication since inhibiting its activity by treatment with brefeldin A (BFA) or

45   Golgicide A (GCA) significantly reduces the yield of infectious viral progeny. Finally, we

46   show that ARF1 dominant negative-mutant T31N over-expression hampered the IBDV

47   infection.

48   Taken together, these results demonstrate that IBDV requires the function of the Rab1b-

49   GBF1-ARF1 axis to promote its replication, making a substantial contribution to the field

50   of birnaviruses-host cell interactions.

51 **IMPORTANCE**

52

53 Birnaviruses are unconventional members of the dsRNA viruses, being the lack of a

54 transcriptionally active core the main differential feature. This structural trait, among others

55 that resemble the plus single-stranded (+ssRNA) viruses features, suggests that birnaviruses

56 might follow a different replication program from that conducted by prototypical dsRNA

57 members and have argued the hypothesis that birnaviruses could be evolutionary links

58 between +ssRNA and dsRNA viruses. Here, we present original data showing the IBDV-

59 induced GC reorganization and the crosstalk between IBDV and the Rab1b-GBF1-ARF1

60 mediated intracellular trafficking pathway. The replication of several +ssRNA viruses

61 depends on the cellular protein GBF1, but its role in the replication process is not clear.

62 Thus, our findings make a substantial contribution to the field of birnaviruses-host cells and

63 provide further evidence supporting the proposed evolutionary connection role of

64 birnaviruses, an aspect which we consider especially relevant for researchers working in the

65 virology field.

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4

67 **INTRODUCTION**

68

69 IBDV, the etiological agent of Gumboro disease, is the best-characterized member of the

70 *Birnaviridae* family, and the prototype of the genus Avibirnavirus (1). Birnaviruses are

71 unconventional double-stranded RNA (dsRNA) viruses, which due to their particular

72 structural and replication features are proposed as an evolutionary link between plus single-

73 stranded RNA (+ssRNA) and dsRNA viruses (2–4). Three-dimensional reconstructions of

74 birnaviruses particles showed that viral capsids are formed by the structural protein VP2,

75 while the genome, the RNA-dependent RNA polymerase (VP1) and the multifunctional

76 protein VP3, are part of transcriptionally-active filamentous structures called

77 ribonucleoprotein complexes (RNPs), located inside the capsids (3, 5). The presence of

78 RNP structures within a dsRNA virus is a unique feature of the *Birnaviridae* family, as

79 these viruses lack the typical transcriptionally active core commonly observed in other

80 dsRNA viruses (3, 6, 7). We have previously shown that after viral particle adsorption and

81 cellular receptor recognition, IBDV hijacks the macropinocytic pathway for entry (8–10).

82 Subsequently, the virus is trafficked into endosomal structures in a Rab5-dependent manner

83 and becomes activated to efficiently infect susceptible host cells after calcium depletion in

84 the endosomal lumen (10). Furthermore, we have demonstrated that IBDV replication

85 machinery, i.e. VP3, VP1, and dsRNA, localize at endosomal compartments and, more

86 importantly, that a functional endocytic pathway is required for the virus to replicate.

87 Additionally, we observed that the endosomal-associated viral replication complexes are

88 located in the juxtanuclear area of the host cell, in close association with the GC, thereby

89 strongly suggesting a functional role of this organelle for IBDV replication (11, 12).

90 The vast majority of +ssRNA viruses replicate associated to host-cell endomembranes,

91 which they modify to serve as hiding niches allowing the virus to avoid the cellular

92 antiviral machinery (13–15). These structures, known as "viral factories", are scaffolds for

93 genome replication and viral morphogenesis (16, 17) where the viral replication machinery

94 is inserted into single or double membrane vesicles that may associate with different

95 organelles like the ER, mitochondria, endo-lysosomal compartment and the GC (13, 15,

96 18). Although some evidence has suggested a link between components of the secretory

97 pathway with the infectious cycle of members of the *Birnaviridae* family, this has not been

98 studied in detail, yet (11, 19, 20).

99 The secretory pathway is an interconnected network of compartments comprising the rough

100 ER, the Endoplasmic Reticulum Exit Sites (ERES), the ER-GC Intermediate Compartment

101 (ERGIC), the GC, and associated trafficking vesicles (21). This pathway is commonly

102 hijacked by viruses to assist them in completing different steps of their replication cycles

103 (22–29). ER-CG anterograde transport occurs through coatomer protein II (COPII) coated

104 vesicles, whereas COPI facilitates the retrograde transport between the GC cisternae to the

105 ER (30). Within this transit, the critical protein Rab1b is loaded at the ERES and GC via

106 direct exchange with the cytosolic pool, remaining only transiently associated with

107 membranes. Once associated with membranes, active Rab1b interacts with a broad set of

108 proteins to orchestrate every step required for both anterograde and retrograde transport

109 (31). Within the cis-GC and ERGIC membranes, Rab1b interacts with the guanine

110 nucleotide exchange factor GBF1 (Golgi-specific BFA resistance factor 1), which in turn

111 catalyzes the activation of the small GTPase ARF1 (ADP-ribosylation factor 1) and

112 promotes the recruitment of the preformed COPI complex to the nascent vesicle (32, 33).

6

113   In a previous work we suggested the secretory pathway involvement in Birnavirus infection

114   (11). In this study, we provide solid evidence showing that IBDV induces the GC re-

115   organization contributing to the localization of the endosome-associated replication

116   complexes. We show that the infection disrupts the GC resulting in a scattered GC

117   distribution around the nucleus without affecting the secretory functionality, measured by

118   Gaussia luciferase secretion. Moreover, we evaluated the role of the Rab1b-GBF1-ARF1

119   machinery in IBDV replication. Rab1b comprises a key regulator of GC transport pathways

120   and we herein demonstrate that knocking-down Rab1b expression by RNA interference and

121   negative-mutant Rab1b-N121I transfection significantly reduces the yield of infectious

122   viral progeny. We show that the catalytic activity of GBF1, a Rab1b effector, is critical for

123   virus replication by using two different pharmacological inhibitors, BFA and GCA. We

124   found that interfering with GBF1 activity causes a dramatic change in the location of viral

125   replication complexes, and negatively impacts on the IBDV infection capacity. Finally, we

126   show that ARF1 negative-mutant T31N over-expression hampered the IBDV infection

127   capacity as well.

128   Taken together, our current findings highlight the functional significance of Rab1b-GBF1-

129   ARF1 mediated cellular transport pathway in IBDV infection.

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7

131 **RESULTS**

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133 **The GC undergoes redistribution during IBDV replication.**

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135 We have previously shown that IBDV RNPs are associated with endosomes, mainly

136 located in the juxtanuclear region of the host cell, close to the GC. Moreover, we observed

137 "cup-shaped structures" formed by VP3 and the mini GC stacks after Nocodazole treatment

138 (Noc, GC fragmentation), and a decreased viral progeny production after BFA treatment

139 (GC disruption) (11, 12). Thus, we hypothesized both the existence of a physical

140 association between VP3-containing endosomes and the GC stacks, and a crucial role of the

141 secretory pathway in the assembly of IBDV (11). To validate the first statement of our

142 hypothesis, we analyzed the GC distribution in IBDV infected cells. QM7 cells were

143 infected at a multiplicity of infection (MOI) of 1 PFU/cell for 24, 36 or 48. Then, we

144 performed indirect immunofluorescence (IIF) using anti-VP3 antibodies and anti-GM130

145 antibodies, to label the IBDV RNPs (red) and the GC (green), respectively. Fig. 1A (panels

146 b and c) depicts a mixed scenario at 24 h p.i. with 46% of the infected cells showing

147 punctuated VP3 at the juxtanuclear region in close association with the GC (panel b), while

148 another fraction (29% of the infected cells) presented VP3 structures peripherally dispersed

149 in the cytosol (panel c). Regarding the GC distribution, a conserved juxtanuclear

150 organization of this organelle in all the infected cells at 24 h p.i. was observed, similarly to

151 the mock-infected situation (panel a). Later in the infection, at 36 h p.i., we observed two

152 additional important changes for both proteins. VP3 was found punctated, GC-associated in

153 27% of infected cells, and in large cytoplasmic structures in 40% of them. On the other

154 side, GM130 lost its normal juxtanuclear distribution in all of the infected cells showing

8

155   GC-associated VP3, to adopt a more scattered one (Fig. 1, images d and e). Finally, at 48 h

156   p.i., the predominant phenotype in 70% of the infected cells was VP3 forming large

157   cytoplasmic structures dispersed throughout the cytoplasm, whereas the GC resumed its

158   original juxtanuclear location (Fig. 1, image f). These observations suggest that the virus

159   usurps and promotes the redistribution of the GC at early stages of infection while leaving

160   this organelle behind once a considerable amount of viral protein has been produced

161   (shown by the large cytoplasmic structures of VP3). To further characterize these

162   observations, two different quantitative analyses were performed. On one side, the number

163   of cells showing normal juxtanuclear (ribbon) versus extended or dispersed GC distribution

164   along the viral infection was determined. Fig. 1B shows the morphology of the GC in cells

165   depicting either phenotype. The bar graph in Fig. 1C shows a marked decrease in infected

166   cells presenting GC ribbon distribution at 36 h p.i. whereas a significant fraction of infected

167   cells recovered the normal GC distribution at 48 h p.i. (black bars). The opposite kinetic is

168   observed for infected cells showing extended or dispersed GC distribution (grey bars). On

169   the other side, a quantitative analysis of VP3-GC association along the viral infection was

170   performed. Fig. 1D shows the percentage of total VP3 structures (punctate and cytoplasmic

171   aggregates) that were GC-associated along the infection, where a significant decrease in the

172   GC-association at 48 h p.i. was observed, in agreement with the images shown in Fig 1A.

173   As mentioned, we previously observed that the endosomal-associated viral replication

174   complexes are located in the juxtanuclear area of the host cell, with a close association with

175   the GC (11, 12). So, to better analyze this point, QM7 cells were transfected with the

176   plasmid encoding EGFP-Rab5 WT and 12 h post-transfection (p.t.) infected with IBDV at

177   an MOI of 1 PFU/cell. At 24 h p.i. the cells were processed for IIF using anti-VP3

178   antibodies and anti-GM130 antibodies to label the IBDV RNPs and the GC, respectively.

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179   While EGFP-Rab5 WT positive endosomes were scattered in the cytoplasm, the RNPs-

180   bearing endosomes were distributed exclusively juxtanuclear, closely associated with the

181   GC fraction (Fig. 1E, images b and c). Besides and surprisingly, the 3D reconstruction

182   analysis of a selected region from the image suggested that the RNPs-bearing endosomes

183   (green signal) mediate the association to the GC, probably through physical contacts (Fig.

184   1E, image d). Thus, to better understand the possible physical association, we performed

185   the same experimental approach described before, with the last 2 h of infection in the

186   presence of Noc to induce the GC dispersion and the distribution of the RNPs-bearing

187   endosomes was analyzed (Fig. 1F). We observed the characteristic juxtanuclear distribution

188   of RNPs-bearing endosomes in DMSO-treated cells, closely associated to the GC (Fig. 1F,

189   images a to e), which was replaced by a dispersed pattern in Noc-treated infected cells, but

190   still associated with the GC remnants (Fig. 1F, images f to j).

191

192   **Nocodazole-induced GC disruption does not disassociate GC-VP3**

193   To deepen this analysis, we incorporated a condition where the last 12 h of the 36 h long-

194   infection was in the presence of the microtubules (MTs) disrupting agent Nocodazole

195   (Noc). MTs participate in maintaining the GC structure, and their depolymerization is

196   known to result in the reorganization of the GC into characteristic ministacks, which appear

197   as punctate structures throughout the cell cytoplasm (34). An MTT [3-(4,5-dimethylthiazol-

198   2-yl)-2,5-diphenyltetrazolium bromide] assay (35) was performed to assess the impact of

199   Noc toxicity on QM7 cells and observed 91% of cell viability after 12 h-long treatment

200   (Fig. 2A). After the incubation time and previous to MTT incorporation, the morphologic

201   aspect of monolayers was documented by Bright Field Microscopy (BFM) (Fig. 2B). In

202   parallel to MTT assays, the functionality of the drug was re-tested by monitoring their well-

203   established impact on GC integrity by Confocal Laser Scanning Microscopy (CLSM) (Fig.

204   2C). We observed that the GC-associated fraction of VP3 remained coupled to the Noc-

205   induced remnants of the GC dispersed throughout the cytoplasm, whereas the large

206   cytoplasmic VP3 structures, not connected to the GC, persisted as observed in the control

207   situation (Fig. 3A,B). These observations point to the existence of a physical association

208   between the RNPs-bearing endosomes and the GC, critical for the juxtanuclear localization

209   of viral replication complexes in infected cells, and strengthen our previous observations

210   suggesting the secretory pathway involvement in virus assembly.

211

212   **IBDV infection induces the GC redistribution without blocking the secretory pathway**

213   **functionality.**

214

215   Our results revealed that IBDV infection induces the transient GC redistribution affecting

216   the architecture of this organelle. To analyze whether this redistribution impacts on its

217   functionality, we assessed the secretion of *Gaussia luciferase* (GLuc) (36). Firstly, GLuc (~

218   20 kDa) and a non-secreted GLuc (~ 50 kDa) protein expression were detected in cell

219   culture media and cell lysates, respectively, using pCMV-GLuc-stably transfected QM7

220   cell line (Fig. 3C). Secondly, to assess the suitability of GLuc secretion for testing a

221   functional secretory pathway, a BFA treatment was performed. BFA is a well-known

222   secretory pathway disruptor (37). After 6 and 24 h of BFA treatment cell lysates and

223   supernatants were collected for immunodetection of GLuc and complete inhibition of GLuc

224   secretion was observed after 6 h of treatment with BFA (Fig. 3D). Finally, GLuc-stably

225   expressing QM7 cells were infected with IBDV at an MOI of 1 PFU/cell and at 24, 36 and

226   48 h p.i. the cell lysates and supernatants were collected for immunodetection of IBDV

11

227   VP3 and GLuc by Western blot technique. A 24 h BFA treatment was included as a control

228   of secretory inhibition. We observed similar levels of secreted GLuc in infected cells

229   compared to mock-conditions for each time point assayed, indicating that the IBDV

230   infection does not affect the functionality of the secretory pathway (Fig. 3E).

231

232   **Inhibition of GBF1 hinders IBDV replication.**

233

234   In recent years, increasing evidence has reported the essential role that GBF1 plays in the

235   replication of several RNA viruses from different families [recently reviewed in (38)].

236   GBF1 is the guanine-exchange factor (GEF) that specifically regulates the COPI-mediated

237   vesicle anterograde transport. Based on its size, GBF1 belongs to the group of large ARF

238   GEFs, which shares a catalytic Sec7 domain that regulates the activation of ARF proteins

239   through binding to the ARF and promoting the exchange of GDP for GTP that leads to the

240   dissociation of the protein from GBF1 (39, 40). So, taking into account the IBDV-induced

241   redistribution of the GC and the role of GBF1 in RNA viruses, we investigated its role in

242   IBDV infection. We assayed the pharmacological compound Golgicide A (GCA), a

243   selective and reversible inhibitor of GBF1 widely used to identify the role of this protein

244   (41). An MTT assay was performed to quantify the impact of BFA and GCA toxicity on

245   QM7 cells viability, which was significatively reduced to 66% in 30 μM GCA-treated

246   compared to DMSO-treated cells (Fig. 2A). Accordingly, the morphologic aspect of GCA-

247   treated monolayers documented by BFM showed significant affectation (Fig. 2B), and, the

248   functionality of the drug was re-tested by monitoring their well-established impact on GC

249   integrity by CLSM (Fig. 2C). Thus, the concentration of GCA was reduced to 10 μM.

250   Then, the cells were infected with IBDV at an MOI of 1 PFU/cell and 24 h p.i. DMSO, 10

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251   or 30 μM GCA, 5μg/ml BFA or 2 μM Noc was added to the infection media for additional

252   12 h of incubation to reach a total of 36 h of infection. We incorporated 10 μM GCA to

253   assess the drug with less impact on cell viability. We employed BFA and Noc as positive

254   and negative internal controls for our experiments, respectively. Whereas BFA treatment of

255   cells leads to the disruption of RNPs-Golgi association, the treatment with Noc produces

256   the pattern mentioned above of viral replication complexes associated with GC ministacks

257   without affecting viral progeny yield (11). Finally, the monolayers were processed by IIF,

258   employing anti-VP3 and anti-GM130 antibodies. As shown in Fig. 4A (image b and the bar

259   graph), DMSO-treated infected cells showed the expected distribution pattern for viral

260   replication complexes, i.e. a marked association of RNPs to the GC in the juxtanuclear

261   region of the cell, quantitatively demonstrated by the 55 ± 6 % of VP3 associated to GC.

262   However, GCA treatment, from a concentration of 10 μM to 30 μM, resulted in a severe

263   disruption of the location of IBDV viral replication complexes, scattered in the cytosol and

264   no longer associated with the GC in the juxtanuclear region, quantitatively demonstrated by

265   the 13 ± 3% and 10 ± 4% of VP3 associated with GC, respectively (Fig. 4A, images c and d

266   and bar graph). On the other hand, and as previously shown (11), Noc treatment led to the

267   dispersion of viral replication complexes that remained associated with the GC ministacks,

268   quantitatively evidenced by the 50 ± 1 % of VP3 associated with GC (Fig. 4A, image e and

269   the bar graph). In contrast, BFA treatment led to a profuse dispersion of viral replication

270   complexes which no longer associate with the GC, similarly to what was observed after

271   GCA treatment of the infected cells, quantitatively demonstrated by the 7 ± 2 % of VP3

272   associated to GC (Fig. 4A, image f and the bar graph). Thus, given the specificity of GCA

273   as a GBF1 inhibitor, these observations strongly suggest a role of this crucial molecule in

274   IBDV infection. Subsequently, to analyze whether the disruption of RNPs localization in

13

275 GBF1-inhibited cells correlated with a decrease in viral infection, we determined the

276 intracellular levels of VP3 in GCA-treated infected cells by Western blot. QM7 cells were

277 mock-treated or infected with IBDV at an MOI of 1 PFU/cell and 24 h p.i. DMSO, 10 or 30

278 µM GCA were added to the infection media and incubated for an additional 12 h to reach a

279 total of 36 h of infection. A significant, dose-dependent decrease in the intracellular level of

280 VP3 in GCA-treated infected cells was observed in comparison with the DMSO-treated

281 control condition (Fig. 4B).

282 Since we demonstrated that GBF1 is a cellular factor required for IBDV replication, we

283 next analysed GBF1 subcellular distribution in IBDV-infected cells using CLSM. Since the

284 antibodies against GBF1 did not recognize the avian protein, we used HeLa cells, which are

285 permissive to IBDV infection and comprises an alternative cellular model in our studies

286 (10–12). As expected, in mock-transfected cells, GBF1 staining was observed in GC-like

287 juxtanuclear structures and in cytoplasmic small dot-like structures (32) (Fig. 4C, left

288 panel). Additionally, similar intracellular GBF1 distribution were observed in

289 IBDV-infected cells (Fig. 4C, right panel), indicating that the major subcellular localization

290 of GBF1 is not modified upon IBDV replication and therefore is likely not recruited to

291 IBDV replication complexes. In addition, GBF1 and VP3 did not co-stain (Fig. 3C, Merge

292 inset in the right panel), strengthening our hypothesis that GBF1 is not recruited to IBDV

293 replication sites. Taken together, our results clearly indicate that the inhibition of GBF1

294 causes a dramatic change in the location of viral replication complexes, and negatively

295 impacts on the IBDV infection capacity.

296

297 **Rab1b is required for IBDV replication**

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298

299   Rab GTPases are essential regulators of most membrane transport events between cellular

300   compartments within eukaryotic cells (42). Rabs cycle between Rab-GDP "inactive" and

301   Rab-GTP "active" forms. Activation of a Rab is coupled to its association with a specific

302   membrane compartment and allows the recruitment of downstream effector proteins (42).

303   Rab1b is loaded independently at the ER exit sites (ERES) interface and GC, via direct

304   exchange with the cytosolic pool, where it interacts with GBF1 to enable the GC-ER

305   retrograde transport (31). So, we investigated the role of Rab1b during IBDV infection.

306   First, we used the plasmid pEGFP-Rab1b WT and pEGFP-Rab1b N122I, a dominant

307   negative mutant (DN) of Rab1b, to over-express the proteins and subsequently analyze

308   their effect on IBDV infection. It has been well-established that over-expression of EGFP-

309   Rab1b WT induces the enlargement of GC and alters the expression levels of certain

310   proteins in mammalian cells (43); whereas over-expression of EGFP-Rab1b N122I blocks

311   the secretory pathway functionality, causing a complete disassembly of the GC (44). Thus,

312   to analyze the phenotype caused by the over-expression of these proteins in our avian

313   cellular model, QM7 cells were transfected with the mentioned plasmids or a pEGFP

314   construct as a control. At 12 h post transfection (p.t.) the monolayers were processed by

315   IIF, employing anti-GM130 antibodies to detect the GC. Fig. 5A shows that, as expected,

316   EGFP-Rab1 WT was associated with vesicles in the juxtanuclear area partly co-localizing

317   with the GC marker GM130, while EGFP-Rab1b N122I presented a cytosolic distribution

318   pattern, accompanied by a profuse GC dispersion (Fig. 5A). Subsequently, to analyze the

319   functional requirements of Rab1b in the IBDV infection cycle, QM7 cells were transfected

320   with the plasmids indicated above and at 12 h p.t. the cells were infected with IBDV at an

321   MOI of 1 PFU/cell. After 24 h of infection, the monolayers were processed by IIF using

15

322 anti-VP3 antibodies to detect the infected cells, and the percentage of transfected-infected

323 cells was determined in each condition. As shown in Fig. 5B, over-expressing EGFP-Rab1b

324 WT resulted in a significant increase in the percentage of infected cells, compared with

325 those over-expressing EGFP. In contrast, we observed a marked decrease in the percentage

326 of infected cells over-expressing the DN mutant, EGFP-Rab1b N122I (Fig. 5B).

327 Subsequently, to confirm the functional role of Rab1b on IBDV infection, we analyzed the

328 effect of its depletion on the viral replication cycle. HeLa cells were transfected with a

329 small interfering RNA (siRNA) designed to decrease the endogenous expression of human

330 Rab1b (44). As indicated before, HeLa cells comprise a non-avian infection model for

331 IBDV, which we and others had successfully employed when molecular tools, such as

332 siRNA or antibodies, were not available for avian cells (10–12, 45, 46). Thus, to ensure

333 high levels of Rab1b depletion, we designed a double hit transfection protocol described in

334 the Material and Methods section. Afterwards, the cells were infected with IBDV at an

335 MOI of 1 PFU/cell or maintained in control medium (mock-infected). At 24 h p.i. the

336 monolayers were processed to verify the Rab1b depletion by Western blot and the

337 supernatants were collected to determine extracellular virus yields. As shown, we observed

338 a marked decrease in both the intracellular level of Rab1b and VP3 in cells transfected with

339 the specific siRNA (Fig. 5C). Accordingly, we observed a significant decrease in viral

340 yields in Rab1b-depleted cells (Fig. 5D). To gain further insights on the dependence of

341 IBDV infection on Rab1b, the sub-cellular distribution of endogenous Rab1b and VP3 at

342 different times p.i. was analyzed. So, HeLa cells were infected with IBDV at an MOI of 1

343 PFU/cell or maintained in control medium (mock-infected). At 24 and 48 h p.i. the cells

344 were processed by IIF technique, using specific anti-VP3 and anti-Rab1b antibodies. As

345 described, Rab1b showed a typical punctate perinuclear distribution pattern in mock-

16

346   infected HeLa cells (47, 48) (Fig. 5E, images a and b). However, in IBDV-infected cells,

347   we observed an evident disruption in Rab1b distribution, whereby Rab1b adopted a

348   disorganized vesicular pattern in the cytoplasm, mainly at late times p.i. (Fig. 5E, images c

349   to j). Also, we observed Rab1b-stained clusters close to cytoplasmic inclusions of VP3

350   (Fig. 5E, images g to j). Finally, to analyze if the observed Rab1b phenotype in infected

351   cells was associated with modifications in the relative levels of Rab1b during IBDV

352   infection, we performed a time course analysis of IBDV infection. HeLa cells were infected

353   with IBDV at an MOI of 1 PFU/cell or maintained in control media (mock-infected). At 24,

354   48 and 72 h p.i. the relative levels of Rab1b were determined by Western blot. As shown in

355   Fig. 5F, the relative level of Rab1b remained constant during the viral infection at the tested

356   time-points of infection. Taken together, our results demonstrate that Rab1b protein has a

357   crucial role in IBDV infection, strongly suggesting that the virus modifies its distribution,

358   maintaining this protein in close association with the assembly sites of newly formed viral

359   particles, without altering its expression profile in host cells.

360

361   **ARF1 is required for IBDV replication**

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363   Rab1b is loaded at the ERES interface and GC where it interacts with GBF1 to enable the

364   GC-ER retrograde transport (31). GBF1, in turn, acts as a guanine nucleotide exchange

365   factor (GEF) for small cellular ADP ribosylation factors (ARFs). ARFs are, essentially,

366   signaling molecules: when GTP-bound ARFs bind GC membranes, they recruit effector

367   proteins that assemble transport carriers [reviewed in (49)]. ARFs have low intrinsic ability

368   to exchange GDP for GTP and require the GEFs to catalyze GDP release. There are six

369   mammalian ARF proteins (ARF1-6). Based on their structural properties, ARFs are divided

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370   into three classes. In human cells, class 1 comprises ARFs 1 and 3, class 2 has ARFs 4 and

371   5, and the most structurally diverse ARF6 is the only member of class 3 (50). GBF1

372   extensively co-localizes with and activates ARF1, 3, 4 and 5 at the ERGIC and the Golgi

373   (51, 52). Among them, ARF1 has been demonstrated to be involved in

374   phosphatidylinositol-4-phosphate (PI4P) production, a lipid essential for GC function and

375   cell viability [reviewed in (53)]. Studies in mammalian systems and in *Saccharomyces.*

376   *cerevisiae* have reported that ARF1 binds to and activates the main enzyme responsible for

377   the PI4P production in the GC, PI4KIIIB (54, 55). So, taking into account previous

378   observations from our lab showing an intrinsic ability of VP3 to bind phosphoinositides

379   (12, 56), we decided to explore ARF1 role in IBDV replication. We used the plasmid

380   pEGFP-ARF1 WT and pEGFP-ARF1 T31N to over-express the proteins and subsequently

381   analyze their effect on IBDV infection. When overexpressed, ARF1 T31N acts as a

382   dominant negative mutant and blocks the intracellular traffic at different steps of the early

383   secretory pathway (57). ARF1 versions were over-expressed as fusion proteins with EGFP

384   in QM7 cells, and at 12 h p.t. the cells were infected with IBDV at an MOI of 1 PFU/cell.

385   After 36 h p.i., the infection was monitored by immunofluorescence (VP3) and the

386   percentage of fluorescent (EGFP) infected cells was quantified. We observed that the over-

387   expression of the ARF1 T31N mutant, which has a BFA-like effect, significantly inhibited

388   IBDV infection, while the WT ARF1 construct showed a similar level of infection

389   compared to EGFP control (Fig. 6A). Having observed such a slight decrease in the

390   infection rate in ARF1 T31N over-expressing cells we decided to strengthen our result by

391   approaching an alternative strategy to test the ARF1 role in the viral infection. Thus we set

392   up a Fluorescence Activated Cell Sorting (FACS) method for determining double-positive

393   cells [EGFP (green)-Alexa 647 immunostaining anti-VP3 signal (far red)]. FACS is a

18

394   powerful technique since it allows the analysis of a population of cells avoiding the

395   observer-subjectivity involved in IF analysis. Thus, EGFP, EGFP-ARF1 WT and pEGFP-

396   ARF1 T31N over-expressing cells were infected as described above, and the monolayers

397   were processed for FACS analysis. Consistently, a slight but significant decrease in IBDV

398   infection was observed when ARF1 functionality was interfered with the overexpression of

399   the dominant-negative version of ARF1, ARF1 T31N (Fig. 6B). Together, these results

400   demonstrate the involvement of ARF1 in IBDV infection.

401

19

402

403 **DISCUSSION**

404

405 Upon infection of a host cell, viruses reorganize intracellular organelles which serve

406 multiple purposes, including the establishment of replication factories or the assembly of

407 infectious virus progeny. Previous data from our laboratory indicate that after viral particles

408 adsorption and cellular receptor recognition, IBDV hijacks the macropinocytic pathway for

409 entry and establishes its replication complexes associated with endocytic vesicles due to the

410 binding of VP3 to the phosphoinositide phosphatidylinositol 3-phosphate (PI3P) present in

411 the cytosolic leaflet of the endosomal membrane (56). Our results also showed that the

412 RNPs-bearing endosomes were located in close proximity to the GC suggesting a physical

413 contact between both organelles (10–12). In this current study, we showed a remarkable

414 reorganization of the GC distribution along with the viral infection (Fig. 1A). The results

415 suggest that the GC is participating in "attaching" the RNPs-bearing structures

416 juxtanuclearly while the viral genome replication and protein translation is taking place and

417 until the viroplasms become viral assembly sites. To elucidate the close association

418 between the RNPs-bearing endosomal compartments and the GC stacks we performed 3D

419 reconstruction analysis of SDCM images and obtained clear images suggesting that the

420 RNPs-bearing endosomes mediate their association to the GC. To confirm this physical

421 link, we performed the same analysis in the presence of a cell microtubules disruptor (Noc)

422 and observed bundles of RNPs-bearing endosomes in association with remnants of GC,

423 indicating that even after the depolymerization of microtubules, these structures remain

424 associated in spite of losing their juxtanuclear localization (Fig. 2A). As mentioned, we

425 have previously shown that VP3 binds to PI3P on the endosomes (56). One particularly

20

426   noteworthy GC lipid is phosphatidylinositol-4-phosphate (PI4P), which contributes to

427   membrane deformation and recruits proteins that form and move transport carriers and is

428   essential for GC function and cell viability [reviewed in (53)]. PI4P in humans is generated

429   through the action of four phosphatidylinositol 4-kinases: PI4KIIa (PI4K2A), PI4KIIb

430   (PI4K2B), PI4KIIIa (PI4KA), and PI4KIIIb (PI4KB) (58–60). PI4KB is localized at the GC

431   and Trans Golgi Network, with PI4P pools in the GC generated by both PI4K2A and

432   PI4KB (61). PI4KB is activated downstream of ARF1 (62). Using *Saccharomyces.*

433   *cerevisiae* as the cellular model, Highland *et al.* (55) recently presented evidence for a

434   model in which ARF1 initiates the final stages of GC maturation by tightly controlling

435   PI4P production through direct recruitment of the kinase complex and a direct ARF1-

436   PI4KB interaction has been shown (55). Thus and given the intrinsic ability of VP3 for

437   binding to PIPs, one hypothesis is that the VP3 could link the endosomes and the GC

438   through its interaction with PI3P on endosomes and PI4P on the GC. In the context of viral

439   infections, the PI4P-rich lipid microenvironment has been demonstrated to be essential for

440   enteroviral and flaviviral RNA replication (63). This hypothesis is supported by our

441   observation regarding the role of ARF1 in the viral infection (Fig.5). Indeed, further studies

442   are necessary to demonstrate the role of PI4P in IBDV viral replication.

443   GBF1 requirement in the IBDV replication cycle is interesting from an evolutionary

444   perspective. Recently a role for GBF1 has been demonstrated for Rotavirus (dsRNA virus

445   belonging to the *Reoviridae* family) assembly (64) and GBF1 has emerged as a host factor

446   mainly involved in the replication of +ssRNA viruses of the families *Picornaviridae* (65–

447   67), *Coronaviridae* (68), *Flaviviridae* (69, 70), *Hepeviridae* (71) and *Togaviridae* (72),

448   reviewed in (38). +ssRNA viruses replicate their genome in the cytoplasm of their host cell

449   in association with cellular membranes. For several +ssRNA viruses, the intracellular

21

450   membranes of the host cells are rearranged during replication, and viral replication

451   complexes are associated with these membrane rearrangements, as we have shown in this

452   and previous studies for IBDV (11, 73). It seems, once again, that birnaviruses are

453   unconventional members among dsRNA. We strongly believe that our results regarding the

454   requirement for GBF1 constitute key findings supporting, together with the lack of a

455   transcriptionally active core inside their single-shelled icosahedral capsid, the hypothesis

456   that birnaviruses could have different evolutionary links with +ssRNA viruses than the

457   other dsRNA viruses characterized to date (3, 5).

458

459   **MATERIALS AND METHODS**

460

461   **Cell lines and culture conditions**

462   Quail muscle fibroblasts (QM7, ATCC CRL-1962) and human epithelial cervix cancer

463   (HeLa, ATCC CCL-2) cell lines were cultured in Dulbecco's modified Eagle's medium

464   (DMEM; catalogue number 12-800058; ThermoFisher Scientific, Argentina) containing

465   10% fetal bovine serum (Gibco FBS: catalogue number 10270-106; ThermoFisher

466   Scientific, Argentina). The culture media was supplemented with penicillin/streptomycin

467   (10,000 U/ml; catalogue number 15140122; ThermoFisher Scientific). Cell lines were

468   grown at 37°C and under a 5% CO_2 atmosphere. Additionally, to provide extra buffering

469   capacity, QM7 cells were cultured in the presence of 40 mM HEPES (catalogue number

470   15630080, ThermoFisher Scientific).

471

472   **Viral stocks production**

473   Serotype I IBDV (Soroa strain) was propagated by employing QM7 cells as previously

474   described by Lombardo and collaborators (Lombardo et al., 1999). Briefly, QM7 cells were

475   grown until 70% confluence and infected with IBDV at an MOI of 0.05 PFU/cell. 72 to 96

476   h post infection (p.i.), the infected cells and the supernatant were collected and exposed to 3

477   freezing/thawing cycles (-80°C 15 min/37°C 5 min) and then clarified by centrifugation at

478   800 x g at 4°C for 15 min. The supernatant was mixed with 20% polyethylene glycol 8000

479   and 3 M NaCl and then incubated for 12 h at 4°C, with gentle shaking. The viral particles

480   were pelleted by centrifugation at 800 x g for 30 min, and the pellet was resuspended in

481   PES buffer [25 mM piperazine-N, N=-bis (2- ethanesulfonic acid) (pH 6.2), 150 mM NaCl,

23

482   and 20 mM CaCl$_2$]. Finally, the viral suspensions were aliquoted in cryovials and stored at -

483   80°C.

484

485   **Antibodies**

486   The following primary antibodies were used: mouse anti-□□-actin (1:2000 for Western

487   blot. Sigma Aldrich, catalogue number A5441), mouse anti-□-actin (1:1000 for Western

488   blot. ThermoFisher, catalogue number MA5-15739), rabbit anti-Rab1b (1:200 for Western

489   blot, 1:100 for IIF, Santa Cruz Biotechnology, catalogue number Sc-599), rabbit anti-VP3

490   (1:1000 for Western blot and 1:500 for IIF, kindly provided by Dr. José F. Rodriguez),

491   rabbit anti-GLuc (1:2000 for Western blot. New England BioLabs, catalog # E8023),

492   mouse anti-GM130 (1:300 for IIF, BD Biosciences, catalogue number 610822), and mouse

493   anti-GBF1 (1:300 for IIF. Biosciences, catalogue number 612116). The following

494   secondary antibodies were used: donkey anti-rabbit Alexa 488 (1:500 for IIF.

495   ThermoFisher Scientific, catalogue number A-21206), goat anti-mouse Alexa 488 (1:500

496   for IFF. ThermoFisher Scientific, catalogue number A-32723), donkey anti-rabbit Cye3

497   (1:500 for IIF. Jackson Immunoresearch, catalogue number 711-165-152), goat anti-mouse

498   Alexa 555 (1:500 for IIF. ThermoFisher Scientific, catalogue number A-32727), goat anti-

499   mouse Alexa 647 (1:500 for IIF. ThermoFisher Scientific, catalogue number A-21247),

500   goat anti-rabbit HRP (1:1000 for Western blot, ThermoFisher Scientific, catalogue number

501   31460), goat anti-rabbit HRP (1:5000 for Western blot. Jackson Immunoresearch, catalogue

502   number A0545), goat anti-mouse HRP (1:10.000 for Western blot. Jackson

503   Immunoresearch, catalogue number A9044).

504

505   **Pharmacological inhibitors**

24

506 Brefeldin A (Millipore-Sigma, catalogue number 20350-15-6), Nocodazole (Millipore-

507 Sigma, catalogue number M1404) and Golgicide A (Sigma Aldrich, catalogue number

508 G0923) were obtained from the indicated providers.

509

510 **Plasmids and transfection methods**

511 A plasmid encoding EGFP-Rab5 WT was kindly provided by Philip D. Stahl (Washington

512 University, St. Louis, MO). Rab5 gene ID: 33418 (also known as AAF51265; BAA88244;

513 CG3664; Dm Rab5; Dmel\CG3664; DmRab5; drab5; dRab5; Drab5; DRab5; l(2)k08232;

514 Rab; Rab-5; rab5; RAB5; RAB5a), (https://www.ncbi.nlm.nih.gov/gene/33418). Plasmids

515 encoding Myc-Rab1b WT and its DN mutant version (Rab1b N121I) were kindly provided

516 by Cecilia Alvarez (CIBICI, UNC-CONICET-Fac. Cs. Químicas, UNC, Córdoba). These

517 plasmids were sub cloned in the vector pEGFP, previously used for a study in our

518 laboratory (44). Rab1b Gene ID: 81876, (https://www.ncbi.nlm.nih.gov/gene/81876).

519 Plasmid encoding EGFP-ARF1 WT and its DN mutant version (ARF1 T31N) were kindly

520 provided by Yves Rouillé (University of Lille, CNRS, Inserm, Institut Pasteur de Lille,

521 Lille, France). Arf1 Gene ID: 375 (https://www.ncbi.nlm.nih.gov/gene/375). The pCMV-

522 GLuc 2 plasmid is a mammalian expression plasmid that constitutively expresses the

523 secreted Gaussia luciferase (GLuc) under the control of the CMV promoter (New England

524 BioLabs, catalog # E8081).

525 For transient transfections, QM7 cells were cultured to reach 80% confluence and

526 transfected using Lipofectamine 3000 (ThermoFisher Scientific, catalogue number

527 L300015) or FuGene HD (Promega, catalogue number E2311), following manufacturer's

528 recommendations. 12 h p.t. the monolayers were fixed with 4% Paraformaldehyde solution

25

529   (PFA) 15 min at room temperature (RT) or infected with IBDV following specific

530   experimental time-points.

531

532   **Viability assay**

533   This assay was performed as described by Kumar *et al.* (74). Briefly, QM7 cells were

534   seeded in a 96-well plate to reach 80% confluency overnight in standard culture conditions.

535   Then, the cells were incubated either for 18 h with 30 μM GCA, 12 h with 2 μM

536   Nocodazole, or 5 μg/ml BFA. Since the three drug stocks were prepared in DMSO, the

537   equivalent amount of this solvent was added to untreated cells for 18 h. After the specified

538   incubation periods, bright field microscopy images were taken and the culture medium was

539   removed and replaced with 100 μl of fresh Phenol red-free DMEM and 10 μl of 5 mg/ml 3-

540   (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium   bromide   (MTT)   stock   solution

541   (prepared in PBS). A negative control of 10 μl of the MTT stock solution added to 100 μl

542   of the medium, without cells, was included. The plate was incubated for 4 h at 37˚C. The

543   formazan produced was solubilized using DMSO as follows. All but 25 μl of the medium

544   was removed from the wells. 50 μl of DMSO were added to each well and mixed

545   thoroughly with a pipette. The plates were incubated for 10 min at 37℃. Each sample was

546   mixed once again, and the absorbance read at 540 and 655 nm. The average value of

547   Abs540 nm from MTT only wells (without cells) was subtracted to all other Abs 540 nm

548   values, which were in turn divided by Abs 655 nm values, to normalize by non-specific

549   background absorbance.

550

551   **Indirect Immunofluorescence**

26

552   QM7 or HeLa cells were seeded on 12 mm coverslips in a 24 multi-well plate. At the

553   indicated time-points post infection (p.i.) or post transfection (p.t.), the cells were washed

554   twice with PBS and fixed with 4% PFA solution for 15 min at RT. Subsequently, the

555   monolayers were permeabilized with 0.05% saponin in PBS containing 0.2% bovine serum

556   albumin (BSA) for 20 min at RT. Then, the monolayers were incubated with primary

557   antibodies for 1 h 30 min at RT or overnight (ON) at 4°C. After extensive washes with

558   PBS, the monolayers were incubated with secondary antibodies for 1 h 30 min at RT.

559   Subsequently, after another round of washes with PBS, the cells were mounted with either

560   Mowiol, Mowiol with Hoechst or Dako fluorescent mounting media with DAPI. Finally,

561   the monolayers were analyzed by LSCM or SDCM. For LSCM analysis, a confocal

562   microscope Olympus FluoView TM FV1000 (Olympus, Argentina) software FV10-ASW

563   (version 01.07.00.16) or a confocal microscope Nikon C1 software EZ-C1 (Nikon, Japón)

564   were employed. Data processing and analysis were performed using Adobe Photoshop CS3

565   (Adobe Systems). For colocalization analysis, we applied ImageJ software. For SDCM

566   analysis, a spinning disc confocal microscope (Quorum Technologies) consisting of an

567   inverted fluorescence microscope (DMI6000B; Leica) equipped with an ORCA-R2 camera,

568   a spinning disc confocal scan head, an ASI motorized XY stage, and a Piezo Focus Drive

569   (Quorum Technologies) were employed. Images were acquired using MetaMorph software

570   (Molecular Devices). Data processing and analysis were performed employing Volocity

571   software (PerkinElmer), and images were processed using Adobe Photoshop and Illustrator

572   (Adobe Systems, Inc.). The acquired images were deconvolved using the calculated point

573   spread function in Volocity. 3D reconstructions were performed employing merged Z-

574   stacks.

575

576 **Flow Cytometry (FACS) analysis**

577 QM7 cells were transfected with EGFP, EGFP-ARF1 WT or EGFP-ARF1 T31N and 12 h

578 p.t. the monolayers were infected with IBDV at an MOI of 1 PFU/cell. At 36 h p.i. the cells

579 were processed for FACS. Cell monolayers were harvested by trypsin treatment and

580 blocked with DMEM 10% FBS before centrifugation of the cells at 75 xg for 5 min.

581 Cellular pellets were washed with PBS and fixed with 4% PFA at RT for 15 min. The cells

582 were permeabilized with 0.05% saponin in PBS containing 0.2% BSA at RT for 20 min,

583 blocked with 15% FBS for overnight at 4ºC, and then incubated with anti-VP3 primary

584 antibodies for 1 h at RT. After washes with PBS, cells were incubated with secondary

585 antibodies conjugated with Alexa Fluor 647 for 1 h, followed by washes in PBS. The cells

586 were suspended in PBS for FACS analysis by using the FACSARIA-III (BD Biosciences)

587 equipped with 488 nm and 633 nm lasers from the Flow Cytometry Facility of the

588 "Facultad de Ciencias Médicas, Universidad Nacional de Cuyo" (Argentina). Data was

589 analyzed using FlowJo 7.6 software.

590 **Quantitative analysis of GC structural changes**

591 The GC morphology in IBDV-infected cells (phenotypes a-f, Fig. 1A) was assessed and

592 categorized as ribbon (juxtanuclear) or extended/dispersed, as exemplified in Fig. 1B. For

593 quantitative determination of GC ribbon or extended phenotype, the distance from the

594 center of the nucleus to the peripheral fringe of the GC was measured in 50 mock-infected

595 cells by using Volocity software (PerkinElmer). The average distance was calculated (9

596 μm) and this value was set as the cut off to score GC phenotype as ribbon or extended. To

597 score the dispersed phenotype, the presence of multiple GC elements was employed as

598 classification criteria. Sixty cells were scored per condition, per trial.

599

600 **SDCM 3D reconstructions**

601 30-40 serial optical sections of each cell at intervals of 0.15 μm approximately were

602 acquired by utilizing the SDCM described before. 3D reconstructions were performed using

603 Volocity software (PerkinElmer), and images were processed using Adobe Photoshop and

604 Illustrator CS3 (Adobe Systems, Inc.).

605

606 **Western blot**

607 Whole cell lysates were prepared using Laemmli Sample Buffer (0.5 M Tris pH 6.8,

608 Glycerol, 10% SDS, 2 mM DTT, and 5% bromophenol blue) and the proteins were

609 denaturalized by heating at 95°C for 10 min. Proteins were resolved by electrophoresis,

610 employing 10, 12 or 15% acrylamide gels, and subsequently transferred to Hybond-ECL

611 nitrocellulose membranes (GE Healthcare). Membranes were washed in PBS and blocked

612 with 5% nonfat milk in PBS, for 2 h at RT or ON at 4°C, and subsequently incubated with

613 the aforementioned primary antibodies ON at 4°C. After three 15 min washes with 0.05%

614 Tween 20 solution in PBS, the membranes were incubated with the corresponding HRP

615 conjugated secondary antibodies for 1 h 30 min at RT. Finally, after extensive washes with

616 0.05% Tween 20 solution in PBS, a chemiluminescent detection kit (WBKLS0100,

617 Millipore or WBLUR0500, Merck-Millipore) was employed to detect immunoreactive

618 bands and the data was collected using a LAS-4000 (Fujifilm) or ChemiDoc ™ XRS +

619 (Biorad) imaging system. The immunoreactive bands were processed and quantified by

620 employing Adobe Photoshop CS5.

621

622 **Viral titration by plaque assay**

29

623   QM7 cells were grown in a 24 multi-well plate until 70% confluence. Then, they were

624   infected with serial dilutions of IBDV. After 1 h of viral adsorption at 37°C, the

625   monolayers were incubated with a 1:1 mix of DMEM 2X and 4% of low melting point

626   agarose aqueous solution, for 5 days at 37°C. Finally, the monolayers were fixed with 10%

627   formaldehyde aqueous solution for 2 h at RT and stained with 1% crystal violet aqueous

628   solution to identify and enumerate lysis plaques for the estimation of viral titers (PFU/ml).

629   For extracellular IBDV titration, the supernatants of infected cells were collected and used

630   for plaque assay as described. For intracellular IBDV titration, infected cells were scrapped

631   in 1 ml of DMEM, and the samples were exposed to 3 freezing/thawing cycles (-80°C 15

632   min/37°C 5 min). Finally, the cellular debris was removed by centrifugation at 1500 x g and

633   the cleared supernatants, containing intracellular viral particles, were employed for plaque

634   assay as described.

635

636   **Knock-down of Rab1b protein**

637   HeLa cells were grown in a 6-well plate until 80% of confluence. The cells were

638   transfected with 20 nM of siRNA against human Rab1b already used in our laboratory for a

639   previous study, whose sequence is CCTTTCTTTGGAACGAGGG (44) (BIONEER,

640   Korea) by employing Lipofectamine 3000, following the manufacturer's recommendations.

641   For the control condition, the cells were incubated in control media. 24 h p.t. (first hit) cells

642   were infected with IBDV at an MOI of 1 PFU/cell for 1 h. Subsequently, the infection

643   media was discarded, and the cells washed with PBS and incubated in fresh media before

644   the second transfection as described (second hit). 24 h p.i. the supernatants were collected

645   for viral titration, employing Reed and Muench technique, and the monolayers were

646   processed for Western blot analysis as described in the Western blot subsection.

30

647

Viral titration by Reed and Muench method

649   Reed and Muench method [50% tissue culture infective dose (TCID50)] was employed for

650   viral titration in Rab1b knock-down experiments exclusively. The methodology was

651   performed as described previously by Delgui and collaborators (11). The cytopathic effect

652   induced by IBDV was detected and quantified after 5 days as described before. The viral

653   titers obtained from three independent experiments were statistically analyzed by a paired

654   Student t-test using Ky-Plot software.

655

Secretion Assay

657   QM7 cells (2×10^4/mL) were seeded in 24-well plates and transfected with pCMV-GLuc

658   using Lipofectamine 3000 Transfection Reagent as indicated by the manufacturer. After 48

659   h, when the monolayers were confluent, the cell culture medium was removed, cells

660   washed twice with PBS, and added at 1 ml per well of selection medium containing

661   DMEM, 10% FBS, and 400 □g/ml of G418 (number ant-gn-5; InvivoGen, USA). The

662   selection medium was replaced every 3 days during a 21-day period increasing the G418

663   until 1.2 mg/ml when non-transfected cells were dead. Then, the stable cell line was

664   expanded accordingly. For the secretion assay, the cells were infected with IBDV (MOI of

665   1) for 24, 36 or 48 h. A set of cells were treated with BFA 100 nmol/L for 24 h. Then, the

666   cell culture medium was removed, and the cells were washed with PBS to remove secreted

667   luciferase and the virus. The fresh medium was added to cell plates, and the medium was

668   collected after each period of incubation, together with a mock condition. Secreted

669   luciferase was detected by immunodetection using antibodies anti-GLuc by Western blot,

670   following the instructions of the manufacturer.

671

Statistical Analysis

Statistical analysis was performed employing the Student's *t* test, using Ky-Plot software

version 2.0 beta 15. Ninety-five percent confidence intervals were used to determine

statistical significance. All data shown are mean ± Standard Deviation (SD) from three

independent experiments. In Flow cytometry experiments, statistical analysis was

performed with one-way analysis of variance (ANOVA), followed by post hoc Tukey Test.

Data were represented as mean ± SEM.

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690

COMPETING INTERESTS

There are no competing interests.

693

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911

912 **FIGURE LEGENDS.**

913

914 **Figure 1. Dynamics of IBDV RNPs-GC association along the IBDV infection. (A)**

915 **Subcellular distribution of GC during IBDV infection.** QM7 cells were infected with

916 IBDV at an MOI of 1 PFU/cell for 24, 36 or 48 h, or maintained in mock condition. The

917 monolayers were processed by IIF to determine the subcellular distribution of GM130 and

918 VP3 proteins and analyzed by SDCM. Images a-f are merges of Z stacks, representatives of

919 three independent assays. Scale bars represent 10 μm. The percentages depicted in images

920 b-f represent the frequency of VP3 phenotypes at 24, 36 and 48 h p.i and are representative

921 of three independent trials where 40 cells were scored per h p.i. **(B-D) Quantitative**

922 **analysis of GC structural changes over the time in IBDV-infected cells.** QM7 cells were

923 infected with IBDV as described for (A). The morphology of the GC in cells with

924 phenotypes a-f from section A were assessed and categorized as ribbon (juxtanuclear) or

925 extended/dispersed as described in Materials and Methods section. A representative image

926 of each CG phenotype is exemplified in the panel B. Scale bar 10 μm. The bar graph in (C)

927 represents the percentage of infected cells displaying corresponding GC phenotypes for

928 each time p.i., and it is representative of two independent trials. The bar graph in (D)

929 represents the percentage of GC-associated VP3 elements (punctate and cytoplasmic

930 aggregates) for each time p.i. and it is representative of two independent trials. 25 cells

931 were scored per condition, per trial. Error bars show SD, $p** \leq 0.05$. **(E) IBDV RNPs-**

932 **endosomes-GC association in IBDV infected cells.** QM7 cells were transfected with

933 EGFP-Rab5wt and at 12 h p.t. the cells were infected as in (A). After 24 h p.i., the

934 monolayers were processed by IIF and analyzed by SDCM as in (A). Images a-c are merges

43

935 of Z stacks, representatives of three independent trials. Scale bar represents10 μm. Image d

936 is a 3D reconstruction of the image c. **(F) Analysis of Noc effect on IBDV RNPs-**

937 **endosomes-GC association.** QM7 cells were treated as described before, and at 22 h p.i

938 were incubated with DMSO or 2 μM Noc for the last 2 h of infection, and processed as in

939 (A). Images a-e are merges of Z stacks of DMSO condition, while images f to j correspond

940 to Noc-treated condition. Yellow arrows indicate IBDV RNPs-endosomes-GC association

941 events. Images are representative of two independent trials. Scale bars represent 10 μm.

942

943 **Figure 2. Viability assay. (A) MTT assay.** QM7 cells were seeded in 96-wells plates to

944 reach 80% confluence overnight. After 24 h in standard culture conditions (37 ℃, 5% CO_2),

945 the cells were incubated either for 18 h with 30 μM GCA, 12 h with 2 μM Noc or 5 μg/ml

946 BFA. As the three drug stocks were prepared in DMSO, equivalent amounts of this solvent

947 were added to untreated cells. After the specified incubation times, the culture medium was

948 removed and replaced with 100 μl of fresh Phenol red-free DMEM. 10 μl of 5mg/ml MTT

949 stock solution (prepared in PBS) were added to each well. A negative control of 10 μl of

950 the MTT stock solution added to 100 μl of medium alone was included. The plate was

951 incubated for 4 h at 37 ° C. The Formazan produced was solubilized using DMSO as

952 follows. All but 25 μl of medium were removed from the wells. 50 μl of DMSO were added

953 to each well and mixed thoroughly with a pipette. The plates were incubated for 10 min at

954 37 ℃. Each sample was mixed again, and the absorbance read at 540 and 655 nm. The

955 average value of Abs540 nm from MTT alone wells (without cells) was subtracted to all

956 other Abs 540 nm values, which were in turn divided by Abs 655 nm values to normalize

957 by nonspecific background absorbance. Error bars show SD, p* ≤ 0.1. **(B) Bright Field**

958 **Microscopy inspection.** Previous to MTT incorporation, the morphologic aspect of

44

959 monolayers was documented by Bright Field Microscopy. **(C) Drugs functionality testing.**

960 Parallel to MTT assay, QM7 cells were seeded in 24-wells plates to reach 80% confluence

961 overnight. After 24 h in standard culture conditions (37℃, 5% CO_2), the cells were

962 incubated either for 18 h with 30 µM GCA, 12 h with 2 µM Noc or 5 µg/ml BFA. Then the

963 monolayers were processed by IIF to determine the subcellular distribution of GM130 (GC

964 integrity) and analyzed by LSCM.

965

966 **Figure 3. IBDV RNPs-GC association in Nocodazole (Noc)-treated infected cells. (A-**

967 **B) Subcellular distribution of GC during IBDV infection.** Two sets of QM7 cells were

968 infected with IBDV at an MOI of 1 PFU/cell for 36 h. To one of them 2 µM Noc was added

969 during the last 12 h of infection, while the other one was DMSO-treated for the same period

970 of time (control condition). The monolayers were processed by IIF to determine the

971 subcellular distribution of GM130 and VP3 proteins and analyzed by SDCM. Images are

972 merges of Z stacks, representatives of three independent trials. Scale bars represent 10 µm.

973 Image on the right panel of (A) is a 3D reconstruction of the inset on the left panel image,

974 obtained as described in the Materials and Methods section. (B) On the upper panels, three

975 images represent typical GC-scattering in infected cells, and the "cup-shaped" structures

976 formed by the GC together with VP3, already described by our laboratory (11). Lower

977 panels are 3D reconstruction of the inset 1 on the upper panels images, obtained as

978 described in the Materials and Methods section. **(C-E) IBDV infection does not block the**

979 **secretory pathway**. QM7 cells were transfected with pCMV-GLuc and a stable cell line

980 was obtained as described in the Materials and Methods section. In **(C)** a cellular lysate and

981 supernatant fraction (SN) was obtained and analyzed by Western blot to detect secreted

982 GLuc (~18 kDa) and non-secreted (NS) GLuc (~50 kDa), by using antibodies anti-GLuc. In

45

983   **(D)** cells were BFA-treated for 6 and 24 h for confirming the GLuc secretion inhibition

984   after secretory pathway disruption. Lysates and SNs of DMSO or BFA-treated cells were

985   obtained after each time point and analyzed by Western blot to detect secreted GLuc (~18

986   kDa) and non-secreted (NS) GLuc (~50 kDa), by using antibodies anti-GLuc. In **(E)** cells

987   were infected with IBDV at an MOI of 1 PFU/cell for 24, 36 or 48 h, or maintained in

988   mock condition, and a separated set of cells were BFA-treated for 24 h to use as a control

989   of secretory function disruption. At each time point, lysates (L) and SNs were collected and

990   analyzed by Western blot to detect VP3 by using antibodies anti-VP3. PonceauS-stained

991   membrane is shown at the bottom (upper panel). A complementary Western blot was

992   performed only with SN samples to detect secreted GLuc (~18 kDa) by using anti-GLuc

993   antibodies. PonceauS-stained membrane is shown at the upper part of the membrane (lower

994   panel). Secretion functionality was determined by quantifying GLuc level in IBDV infected

995   SNs and in mock-infected SNs and obtaining the corresponding ratio (GLuc IBDV/GLuc

996   Mock) for each time point. Non-significant differences were obtained.

997

998   **Figure 4. GBF1 requirement in IBDV infection. (A) Involvement of GBF1 in RNPs-**

999   **GC association.** QM7 cells were infected with IBDV at an MOI of 1 PFU/cell or

1000   maintained in control media. At 24 h p.i. the monolayers were incubated with DMSO; 10 o

1001   30 µM GCA; 5µg/ml BFA or 2 µM Noc until 36 h p.i. The monolayers were processed by

1002   IIF and analyzed by SDCM as described before. Images a to f correspond to merges of Z

1003   stacks, representatives of three independent trials. Scale bars represent 10 µm. The bar

1004   graph represents the percentages of VP3 punctate associated with GC and they are

1005   representative of three independent trials. Total VP3 punctate, and VP3 GC-associated

1006   punctate were scored by employing ImageJ software and the percentage of GC-associated

46

1007   VP3 punctate calculated for twenty cells, per condition per trial. **(B) GBF1 role in IBDV**

1008   **infection.** QM7 cells were infected as in (A) and 24 h p.i. cells were incubated with

1009   DMSO, 10 or 30 μM GCA until 36 h p.i. Subsequently, the monolayers were processed for

1010   Western blot to determine the intracellular level of VP3 protein, as described in the

1011   Materials and Methods section. Western blot image corresponds to an experiment

1012   representing three independent trials, and the complete data is shown in the normalized bar

1013   graph. Error bars show SD, $p^* \leq 0.1$, $p^{**} \leq 0.05$. **(C) Subcellular distribution of**

1014   **endogenous GBF1 during IBDV infection.** HeLa cells were infected with IBDV at an

1015   MOI of 1 PFU/cell or maintained in control media. At 36 h p.i. the monolayers were

1016   processed by IIF to determine the subcellular distribution of GBF1 and VP3 and analyzed

1017   by LSCM. Images are representative of three independent trials. Scale bars represent 20

1018   μm.

1019

1020   **Figure 5. Rab1b activity is required in IBDV infection. (A) Phenotypic analysis of**

1021   **EGFP-Rab1b WT and its DN mutant form over-expression in avian cells**. QM7 cells

1022   were transfected with EGFP, EGFP-Rab1b WT or EGFP-Rab1b N121I and 12 h p.t were

1023   processed by IIF to stain the GC by employing anti-GM130 antibodies. Finally, the

1024   monolayers were analyzed by SDCM. Bar scales represent 10μm. **(B) Effect of Rab1b WT**

1025   **and its DN mutant overexpression in IBDV infection.** QM7 cells were transfected with

1026   EGFP, EGFP-Rab1b WT or EGFP-Rab1b N121I and 12 h p.t the monolayers were infected

1027   with IBDV at an MOI of 1 PFU/cell. At 24 h p.i. the cells were processed by IIF as

1028   described before and analyzed by SDCM. A representative image of transfected-infected

1029   cells in each condition is shown, and the data represented in the normalized bar graph

1030   correspond to three independent trials. Scale bars represent 10 μm. Error bars show SD,

47

1031   p**<0, 05. **(C-D) Rab1b requirement for the generation of the IBDV progeny.** HeLa

1032   cells were subjected to a double hit transfection protocol with siRNAs against human

1033   Rab1b. First, the monolayers were transfected with Rab1b siRNAs (first hit) and at 24 h p.t,

1034   the monolayers were infected with IBDV at an MOI of 1 PFU/cell or maintained in control

1035   media. Immediately after viral adsorption, the culture media was replaced, and the cells

1036   transfected again with the Rab1b siRNAs (second hit). At 24 h p.i. the monolayers were

1037   processed for Western blot to verify the knock-down of Rab1b and the intracellular level of

1038   VP3. Western blot images shown in C correspond to an experiment of three independent

1039   trials. The quantitative data in the normalized bar graphs correspond to three independent

1040   trials. Error bars show SD, $p** \leq 0.05$. In **(D)** supernatants of infected cells were employed

1041   to determine the extracellular viral yields as described in the Material and Methods section.

1042   Error bars show SD, $p*** \leq 0.001$. **(E) Subcellular distribution of endogenous Rab1b**

1043   **during IBDV infection.** HeLa cells were infected with IBDV at an MOI of 1 PFU/cell or

1044   maintained in control media. At 24 and 48 h p.i. the monolayers were processed by IIF to

1045   determine the subcellular distribution of Rab1b and VP3 and analyzed by LSCM. Images

1046   are representative of three independent trials. Scale bars represent 10 μm. **(F) Kinetic of**

1047   **Rab1b accumulation in IBDV infection.** HeLa cells were infected with IBDV at an MOI

1048   of 1 PFU/cell or maintained in control media. At 24, 48 and 72 h p.i. the monolayers were

1049   processed for Western blot to track the Rab1b intracellular level, as described in the

1050   Materials and Methods section. Western blot image and the bars graph correspond to one

1051   representative experiment.

1052

1053   **Figure 6. ARF1 requirement in IBDV infection. (A) Effect of ARF1 WT and its DN**

1054   **mutant overexpression in IBDV infection by LSCM.** QM7 cells were transfected with

1055 EGFP, EGFP-ARF1 WT or EGFP-ARF1 T31N and 12 h p.t the monolayers were infected

1056 with IBDV at an MOI of 1 PFU/cell. At 24 h p.i. the cells were processed by IIF as de-

1057 scribed before and analyzed by LSCM. A representative image of transfected-infected cells

1058 in each condition is shown, and the data represented in the normalized bar graph corre-

1059 spond to three independent trials. Scale bars represent 10 μm. Error bars show SD, p**<0,

1060 05. **(B) Effect of ARF1 WT and its DN mutant overexpression in IBDV infection by**

1061 **Flow cytometry (FACS)**. QM7 cells were transfected with EGFP, EGFP-ARF1 WT or

1062 EGFP-ARF1 T31N and 12 h p.t the monolayers were infected with IBDV at an MOI of 1

1063 PFU/cell. At 24 h p.i. the cells were processed by FACS as described in the Materials and

1064 Methods section. Representative FACS histograms from two independent trials show the

1065 mean fluorescence intensity (MFI) in relative units for each condition: GFP, ARF WT or

1066 EGFP-ARF1 T31N. Dotted and solid lines represent mock and infected conditions, respec-

1067 tively. (C) Bars graph shows MFI for each condition with a One-Way ANOVA statistical

1068 analysis, followed by a Tukey test. Bars express the mean +/- the standard error of the me-

1069 dia (SEM) of two independent trials. *P< 0.5.

A

B

Bright Field Microscopy

C

Drugs Functionality Test - Confocal Laser Scanning Microscopy

Untreated   DMSO 18 h   GCA 18 h   BFA 12 h   NOC 12 h

GC integrity

A GM130-DAPI-VP3(IBDV)

QM7 DMSO — IBDV 36 h p.i.

B QM7 Noc — IBDV 36 h p.i.

1 INSET / 2 INSET / 3D INSET (1)

C MWM Lysate SN
130 kDa
70 kDa
55 kda
NS GLuc
35 kDa
25 kDa
GLuc
15 kDa

D 6 h / 24 h
DMSO BFA DMSO BFA
MWM Lysate SN Lysate SN Lysate SN Lysate SN

E 24 h / 36 h / 48 h
BFA Mock IBDV Mock IBDV Mock IBDV
MWM L SN L SN L SN L SN L SN L SN
130 kDa
70 kDa
55 kda
35 kDa → IBDV VP3
Ponceau S
25 kDa
15 kDa

24 h / 36 h / 48 h
BFA Mock IBDV Mock IBDV Mock IBDV
MWM
25 kDa
Ponceau S
15 kDa
→ GLuc

0.98 ± 0.08   1.00 ± 0.07   0.92 ± 0.05   GLuc IBDV/ GLuc Mock

A QM7 36 h p.i.

a — Mock
b — IBDV + DMSO
c — IBDV + 10 μM GCA
d — IBDV + 30 μM GCA
e — IBDV + 2 μM Noc
f — IBDV + 5 μg/ml BFA

Nuclei-GM130-VP3

INSET

GC-associated VP3 puncta (%)

DMSO — 10 μM GCA *** — 30 μM GCA *** — 2 μM Noc ns — 5 μg/ml BFA ***

B QM7 36 h p.i.

DMSO | 10 μM GCA | 30 μM GCA
− + | − + | − + IBDV

→ Actin
→ VP3

VP3/Actin

DMSO | 10 μM GCA * | 30 μM GCA **

C HeLa 36 h p.i.

Mock | IBDV

GBF1 | GBF1 — VP3 — Merge

A

QM7

IBDV (VP3)

EGFP | EGFP-ARF1 WT | EGFP-ARF1T31N

% of transfected and infected cells (normalized)

ns
**

EGFP | EGFP-ARF1 WT | EGFP-ARF1 T31N

B

☐ Mock-infected
▨ IBDV-infected

Number of Eventss

M.F.I. (R.U.)

EGFP | EGFP-ARF1 WT | EGFP-ARF1 T31N

1x10⁴   5x10⁴

M.F.I. (R.U.)

n.s.
*

EGFP | EGFP-ARF1 WT | EGFP-ARF1 T31N