1 **Rab3a, a small GTP-binding protein, stabilizes the murine leukemia virus Gag protein**

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21 **ABSTRACT**

22 CD63, a tetraspanin protein, is involved in virion production in human immunodeficiency virus (HIV-

23 1). We had recently reported that Rab3a, a small GTP-binding protein, interacts with and enhances

24 lysosomal degradation of CD63 and that Rab3a-free CD63 is incorporated into HIV-1 particles. In

25 this study, we analyzed impact of CD63 and Rab3a on virion production and infectivity of released

26 virions of murine leukemia virus (MLV), which is also a member of the retrovirus family. We found

27 that CD63 was incorporated into MLV particles. CD63 silencing, mediated by an shRNA, decreased

28 the infectivity of released MLV particles but did not decrease virion production, suggesting that

29 incorporation of CD63 into MLV particles is a prerequisite for efficient MLV infection. Rab3a

30 silencing significantly reduced the amount of MLV Gag protein in cell lysates and induced lysosomal

31 degradation of the MLV Gag protein. Recovery of Rab3a expression restored Gag protein expression.

32 The MLV Gag protein interacted with Rab3a and interfered with Rab3a-mediated lysosomal

33 degradation of CD63. These results show that association between the MLV Gag protein and Rab3a

34 is required for stability of the MLV Gag protein; it also inhibits Rab3a-mediated lysosomal

35 degradation of CD63, which potentiates MLV infection.

36

37 **IMPORTANCE** Murine leukemia virus belongs to the *Retroviridae* family and is frequently used in

38 mouse models of human diseases, such as leukemia, and as a vesicle to transfer genes of interest into

39 target cells. Understanding underlying molecular mechanisms of murine leukemia virus replication

40 will lead to development of more efficient cell-type-specific murine leukemia virus vectors. We

41 recently reported that CD63 is involved in virion production in HIV-1, another retrovirus, and that

42 Rab3a interacts with and enhances lysosomal degradation of CD63. In the present study, we analyzed

43 roles of CD63 and Rab3a in murine leukemia virus replication. It was found that incorporation of

44 CD63 into murine leukemia virus particles is required for efficient infection by released murine

leukemia virus particles. We also found that the murine leukemia virus Gag protein interacts with Rab3a to inhibit lysosomal degradation of the Gag protein and Rab3a-mediated degradation of CD63.

Keywords

CD63, tetraspanin, Rab3a, small GTP-binding protein, murine leukemia virus

51 **Introduction**

52 The replication mechanism of human immunodeficiency virus type 1 (HIV-1) has been studied in

53 detail. Tetraspanin family proteins form special microdomains in the plasma membrane, named

54 tetraspanin-enriched microdomains (TEMs); these microdomains are involved in many biological

55 events. Tetraspanin proteins are known to participate in HIV-1 replication. CD63, a tetraspanin family

56 member, is involved in HIV-1 entry into host cells. CD63 silencing by a specific shRNA in host cells

57 inhibits HIV-1 infection (1,2). CD63 disrupts the trafficking of CXCR4 to the plasma membrane and

58 inhibits CXCR4-tropic HIV-1 infection (3).

59 CD63 is also involved in virion production in HIV-1. HIV-1 particles are formed on TEMs of HIV-1-

60 producing cells (4). Although CD63 is specifically localized to late endosomes/lysosomes and HIV-1

61 particles are formed in plasma membranes, CD63 is preferentially incorporated into HIV-1 particles

62 (5), suggesting that CD63 plays a role in HIV-1 virion formation. CD63 silencing inhibits HIV-1 virion

63 production (6-8). Previously, we have also shown that gamma-interferon-inducible lysosomal

64 thiolreductase restricts HIV-1 virion production by digesting disulfide bonds of CD63 (9) and that a

65 CD63 mutant with serine substitutions at conserved cysteine amino acid residues (CD63 TCS) also

66 suppresses HIV-1 virion production.

67 Recently, we demonstrated that CD63 interacts with Rab3a, a small GTP-binding protein, to induce

68 degradation of CD63 (8). Although CD63 is efficiently incorporated into HIV-1 particles and binds to

69 Rab3a, the Rab3a protein was not detected in virion-containing fractions, which suggests that only

70 Rab3a-free CD63 is incorporated into HIV-1 particles. Rab3a overexpression or silencing had a

71 moderate inhibitory effect on HIV-1 virion production. These results indicate that Rab3a does not have

72 a direct, critical role in HIV-1 virion production. However, other members of the Rab family, including

73 Rab7a (10), Rab9 (11), and Rab27a (12), are required for HIV-1 virion production. In addition, Rab11-

74 FIP1C (13) and Rab14 (14) induce incorporation of the HIV-1 amphotropic envelope glycoprotein

75 (Env) complex into the virus particles.

76 Murine leukemia virus (MLV), another member of the retrovirus family, induces leukemia (15),

77 immunodeficiency (16), or neurological disease (17) in susceptible mice and is, therefore, used in

78 mouse models of human disorders. MLV is also utilized as a vesicle to transfer genes of interest into

79 target cells in many biological fields (18). Thus, MLV is one of the most important subjects not only

80 in medical biology but also in many other areas of biology. However, roles of CD63 and Rab3a in

81 MLV replication remain unclear.

82 In this study, we analyzed effects of CD63 and Rab3a on MLV virion production. We found that CD63

83 silencing decreased the infectivity of released MLV particles but did not decrease MLV virion

84 production, indicating that CD63 is required for efficient MLV infection. Rab3a silencing significantly

85 reduced the MLV Gag protein level and virion production, indicating that Rab3a is a critical cellular

86 factor in MLV replication.

87 **Results**

88 **CD63 is incorporated into MLV particles.** It has already been demonstrated that CD63 is

89 incorporated into HIV-1 particles (5). To examine whether CD63 is similarly incorporated into MLV

90 vector particles, 293T cells were transfected with C-terminally GFP-tagged CD63 (CD63-GFP) using

91 an expression plasmid with or without MLV vector construction plasmids. Cell lysates and virion

92 pellets were analyzed by Western immunoblotting. The CD63-GFP protein was detected in the virion

93 pellets only when the 293T cells were co-transfected with the MLV vector construction plasmids (Fig.

94 1). In the absence of MLV vector construction plasmids, the CD63-GFP protein was detected in the

95 cell lysate but not in the virion-containing fraction. These results support conclusions of previous

96 research, i.e., CD63-GFP is incorporated into MLV particles (19).

97

98 **CD63 did not inhibit MLV Env-mediated infection.** It has previously been reported that

99 incorporation of CD63 into HIV-1 particles inhibits HIV-1 Env-mediated infection (20). To assess

100 whether CD63 also inhibits MLV Env-mediated infection, 293T cells were transfected with

101 amphotropic MLV vector construction plasmids together with empty plasmids or CD63-GFP

102 expression plasmids. Culture supernatants from the transfected cells were inoculated into human

103 TE671 cells to estimate transduction titers. Transduction titers were similar in the presence and

104 absence of CD63-GFP (Fig. 2A). MLV Gag p30 levels in the cell lysates and virion fractions were

measured by Western immunoblotting. p30 levels were unchanged by CD63-GFP expression (Fig. 2B). These results show that exogenous CD63 expression does not inhibit amphotropic Env-mediated infection or MLV virion production.

CD63 is required by the MLV Env protein for efficient infection. The need for CD63 for efficient HIV-1 virion production is well documented (6-8). To examine whether CD63 is also required for MLV particle production, a lentiviral vector encoding an shRNA against CD63 mRNA (shCD63) was constructed and inoculated into TE671 cells that endogenously expressed CD63 at a relatively high level (8). The control and shCD63-expressing TE671 cells were transfected with amphotropic MLV

vector construction plasmids and the culture supernatants were inoculated into TE671 cells to determine transduction titers. shCD63 significantly decreased transduction titers (Fig. 3A). CD63, MLV Gag, and Env protein levels in the cell lysates and virion fractions were analyzed by Western immunoblotting. The endogenous CD63 levels were reduced in the shCD63-transduced cells (Fig. 3B), confirming that CD63 was silenced. MLV Gag and Env protein levels in the cell lysates and virion fractions were unchanged. These results indicate that endogenous CD63 expressed in MLV vector-producing cells is required for efficient infection by released MLV particles but not for MLV virion production. CD63 is, therefore, incorporated into MLV vector particles, which may potentiate infectivity.

In the experiments detailed above, a replication-defective MLV vector was used. Next, we analyzed effects of endogenous CD63 on MLV virion production using the replication-competent Moloney MLV. TE671 cells artificially expressing an ecotropic MLV receptor (TE671-mCAT1) were inoculated with replication-competent Moloney MLV (21). The Moloney MLV-producing cells were then transduced by an shCD63-encoding or empty lentiviral vector and selected using puromycin. Viral titers of their culture supernatants were measured using the XC cell test (22). Viral titers were decreased by transduction of shCD63 (Fig. 3C). The endogenous CD63 level was also reduced by shCD63, confirming that CD63 was silenced (Fig. 3D). The amounts of Gag protein in the cell lysates and virion fractions were not altered. These results support the conclusion that endogenous CD63

132 expressed in MLV-producing cells is required for efficient infection by released MLV particles.

133

134 **Endogenous Rab3a is required for Gag protein expression.** Recently, we reported that Rab3a binds

135 to CD63 to induce degradation of CD63 and that Rab3a-free CD63 is incorporated into HIV-1 particles

136 (8). To examine whether Rab3a is involved in MLV vector production, 293T cells were inoculated

137 with a lentiviral vector encoding shRNA against Rab3a mRNA (shRab3a) and selected using

138 puromycin. We discovered that the endogenous Rab3a level was indeed reduced because of shRab3a

139 (Fig. 4A). Control and shRab3a-expressing 293T cells were transfected with amphotropic MLV vector

140 construction plasmids. Culture supernatants from the transfected cells were inoculated into TE671

141 cells to estimate transduction titers. Transduction titers were reduced to <1% by Rab3a silencing (Fig.

142 4B). To determine whether this transduction titer reduction due to Rab3a silencing is induced by a

143 decrease in MLV Gag protein expression, Western immunoblotting was performed using anti-MLV

144 Gag p30 antibodies. The amount of Gag protein in the cell lysates and virion fractions also

145 significantly decreased (Fig. 4C). However, when the cells were transfected with HIV-1 vector

146 construction plasmids, the HIV-1 Gag protein levels were unchanged by Rab3a silencing (8). When

147 replication-competent Moloney MLV-producing TE671-mCAT1 cells were inoculated with the

148 shRab3a-encoding lentiviral vector, the Rab3a protein level decreased (Fig. 4D). Rab3a silencing

149 reduced the MLV Gag p30 level in the cell lysates. Viral titers of the culture supernatants were reduced

150 to <1% by Rab3a silencing (Fig. 4E). These results show that endogenous Rab3a is critical for MLV

151 Gag protein expression.

152 To obtain more data for confirming this conclusion, we constructed another C-terminally HA-tagged

153 Rab3a expression plasmid resistant to shRab3a-mediated silencing, named Rab3a RS-HA (8).

154 Compared with wild-type Rab3a, the Rab3a RS-HA expression plasmid has synonymous nucleotide

155 substitutions but no amino acid changes in its shRab3a target sequence. Control and shRab3a-

156 expressing 293T cells were transfected with amphotropic MLV vector construction plasmids together

157 with empty or Rab3a RS-HA expression plasmids. Transduction titers of culture supernatants from

158 the Rab3a-silenced cells were much lower than those of culture supernatants from the control cells

159 (Fig. 5A), as mentioned above (Fig. 4B). Transduction titers were recovered by Rab3a RS-HA

160 expression. Similarly, MLV Gag p30 levels were reduced by Rab3a silencing and were recovered by

161 Rab3a RS-HA expression (Fig. 5B). The exogenous Rab3a RS-HA expression in control 293T cells

162 moderately attenuated the transduction titers, but did not affect MLV Gag protein expression. These

163 results support the conclusion that Rab3a is required for MLV Gag protein expression.

164

165 **The MLV Gag protein is degraded in lysosomes in the absence of Rab3a.** MLV Gag protein levels

166 were greatly reduced in Rab3a-silenced cells. To examine whether the Gag protein is degraded in the

167 lysosome or proteasome of Rab3a-silenced cells, Rab3a-silenced 293T cells were transfected with

168 MLV Gag-Pol expression plasmids and treated with inhibitors of lysosome (concanamycin A; CMA)

169 or proteasome (MG-132). CMA treatment resulted in elevated MLV Gag protein levels, but this was

170 not observed in the case of MG-132 treatment (Figs. 6A and B). These results show that endogenous

171 Rab3a inhibits lysosomal degradation of the MLV Gag protein.

172

173 **MLV increased CD63 levels.** We reported that Rab3a decreases CD63 expression levels by inducing

174 degradation of CD63 (8). During this study, we noticed that the CD63 level was unchanged by Rab3a

175 in the presence of a MLV vector. To examine whether the MLV Gag protein suppresses Rab3a-

176 mediated degradation of CD63, 293T cells were transfected with CD63-GFP and C-terminally HA-

177 tagged wild-type Rab3a (Rab3a WT-HA) expression plasmids together with pcDNA3.1 or MLV Gag-

178 Pol expression plasmids. Cell lysates prepared from transfected cells were analyzed by Western

179 immunoblotting. Similar to the results of previous reports (8), exogenous Rab3a WT-HA expression

180 decreased CD63-GFP levels in the absence of MLV Gag-Pol expression plasmids (Fig. 7A). However,

181 in the presence of Gag-Pol expression plasmids, the CD63-GFP level was not changed because of

182 Rab3a WT-HA. These results indicate that the MLV Gag-Pol protein suppresses Rab3a-mediated

183 reduction of CD63 levels.

184 This result prompted us to speculate that endogenous CD63 levels are increased in MLV-infected cells.

185 To test this hypothesis, endogenous CD63 levels in control TE671-mCAT1 cells and replication-

competent Moloney MLV-producing TE671-mCAT1 cells were measured by Western immunoblotting. As expected, the endogenous CD63 level in the Moloney MLV-producing cells was higher than that in control cells (Fig. 7B). This result supports the conclusion that MLV increases CD63 levels.

MLV Gag protein binds to Rab3a. To examine whether the MLV Gag protein binds to Rab3a, 293T cells were transfected with Rab3a WT-HA expression plasmids together with pcDNA3.1 or MLV Gag-Pol expression plasmids. The MLV Gag protein in cell lysates prepared from transfected cells was precipitated using goat anti-MLV p30 antibodies, and the precipitates were analyzed by Western

195 blotting using anti-HA antibodies. The Rab3a WT-HA protein was detected in the presence of MLV

196 Gag-Pol expression plasmids, but not in their absence (Fig. 8A). This result shows that the MLV Gag

197 protein binds to Rab3a WT-HA.

198 We analyzed cellular localization of the MLV Gag and Rab3a WT-HA proteins. 293T cells were

199 transfected with MLV Gag-Pol and Rab3a WT-HA expression plasmids and permeabilized with

200 methanol. The cells were treated with goat anti-MLV p30 and rabbit anti-Rab3a antibodies, then with

201 PI-conjugated anti-goat IgG and FITC-conjugated anti-rabbit IgG antibodies. In almost all cells, MLV

202 Gag and Rab3a proteins showed equal distribution in the cytoplasm (Supplementary Fig. 1A).

203 However, Rab3a WT-HA and MLV Gag proteins were co-localized at the top of the cell. Furthermore,

204 when the Rab3a WT-HA protein was detected in the left side of a cell, the Gag protein was also

205 concentrated in the same region (Supplementary Fig. 1B). These results indicate that the MLV Gag

206 protein co-localizes with Rab3a.

207 We have previously reported that Rab3a interacts with CD63 to induce degradation of CD63. To

208 examine whether the MLV Gag protein inhibits interaction between CD63 and Rab3a, 293T cells were

209 transfected with CD63-GFP and Rab3a-HA expression plasmids together with pcDNA3.1 or MLV

210 Gag-Pol expression plasmids. The CD63-GFP protein in cell lysates prepared from the transfected

211 cells was precipitated using anti-GFP antibodies, and the precipitates were analyzed with anti-HA

212 antibodies. The Rab3a WT-HA protein was detected in cells transfected with pcDNA3.1, but not in

213 the presence of MLV Gag-Pol expression plasmids (Fig. 8B). This result shows that the MLV Gag-

214 Pol protein competes with CD63 to bind with Rab3a, thereby inhibiting Rab3a-mediated degradation

215 of CD63.

216

217 **Active and inactive forms of Rab3a decrease and increase MLV vector infectivity, respectively.**

218 Rab3a is a small GTP-binding protein. The GTP- and GDT-binding forms of Rab3a are active and

219 inactive, respectively. Rab3a containing an asparagine substitution at its threonine amino acid residue

220 36 (T36N) cannot bind to GTP and therefore, functions only as a constitutively inactive mutant (23).

221 Another Rab3a mutant containing a leucine substitution at its glutamine amino acid residue 81 (Q81L)

222 lacks GTPase activity and functions only as a constitutively active mutant (24,25). To assess impacts

223 of these forms of Rab3a on MLV vector production and/or infectivity, C-terminally HA-tagged T36N

224 and Q81L Rab3a mutant expression plasmids (Rab3a T36N-HA and Rab3a Q81L-HA, respectively)

225 were constructed (8). 293T cells were transfected with amphotropic MLV vector construction

226 plasmids together with empty, Rab3a WT-HA, T36N-HA, or Q81L-HA expression plasmids. Culture

227 supernatants from the transfected cells were inoculated into TE671 cells to estimate transduction titers,

228 which were decreased by Rab3a WT-HA and Q81L-HA but increased by Rab3a T36N-HA (Fig. 9A).

229 However, MLV Gag protein levels in the cell lysates and virion fractions were unchanged (Figs. 9B

230 and C). These results show that the active form of Rab3a inhibits the infectivity of released MLV

231 vectors and conversely, the inactive form enhances infectivity. HA-tagged Rab3a proteins were easily

232 detected in the virion fraction, suggesting that Rab3a protein is incorporated into MLV particles,

233 unlike HIV-1 particles (8). This result supports the conclusion that the Rab3a protein binds to the MLV

234 Gag protein.

235

236 **Mouse Rab3a binds to the MLV Gag protein.** The natural host of MLV is the mouse. To examine

237 whether mouse Rab3a is also necessary for MLV Gag protein expression, a lentiviral vector encoding

238 shRNA against mouse Rab3a was inoculated into mouse NIH3T3 cells. No puromycin-resistant

239 colonies were observed. Compared with human Rab3a, mouse Rab3a contains two amino acid

240 substitutions in its C-terminal region (Supplementary Fig. 2). To assess whether the mouse Rab3a

241 protein interacts with the MLV Gag protein, these amino acid substitutions were introduced into the

242 Rab3a RS-HA expression plasmid (mRab3a RS-HA). 293T cells were transfected with this mRab3a

243 RS-HA plasmid together with pcDNA3.1 or MLV Gag-Pol expression plasmids. The MLV Gag

244 protein was precipitated using anti-MLV p30 antibodies. The precipitates were analyzed by Western

245 immunoblotting using anti-HA antibodies. The mRab3a RS-HA protein was only detected in the

246 presence of the MLV Gag protein (Fig. 10A), indicating that the mRab3a RS-HA protein interacts

247 with the MLV Gag protein.

248 To determine whether mRab3a RS-HA stabilizes the MLV Gag protein, control or Rab3a-silenced

293T cells were transfected with amphotropic MLV vector construction plasmids together with pcDNA 3.1 or mRab3a RS-HA expression plasmids. Culture supernatants from the transfected cells were inoculated into 293T cells, transduction titers were measured, and cell lysates prepared from the transfected cells were analyzed by Western immunoblotting using anti-p30, -HA, and -actin antibodies. mRab3a RS-HA increased transduction titers (Fig. 10B) and p30 levels (Fig. 10C) in Rab3a-silenced cells, showing that mRab3a RS-HA elevates MLV Gag protein levels and transduction titers when Rab3a expression is silenced. These results suggest that mouse Rab3a also binds and stabilizes the MLV Gag protein.

Streptolysin O has no effect on MLV virion production. It has been reported that Rab3a induces transport of lysosomes to the plasma membrane during the repair of plasma membrane pores caused by streptolysin O (SLO) (26,27). Therefore, we speculated that Rab3a-mediated lysosome transport is involved in MLV particle production and that SLO plasma membrane injury enhances particle production. To test this hypothesis, 293T cells transfected with amphotropic MLV vector construction plasmids were treated with SLO for 10 min, washed with PBS, and then cultured in fresh medium for 24 h. Gag p30 levels in the cell lysates and virion pellets were not changed by SLO treatment (Supplementary Fig. 3A). Transduction titers were reduced by treatment with 50-ng/ml SLO (Supplementary Fig. 3B). Treatment with 100-ng/ml SLO resulted in death of many of the transfected

267 cells. This suggests that Rab3a-mediated lysosome transport during plasma membrane repair is not

268 related to MLV virion production.

269

Discussion

In this study, we found that Rab3a is essential for MLV Gag protein expression and that interaction between Rab3a and the Gag protein inhibits Rab3a-mediated degradation of CD63 that is required for efficient infection by released MLV particles. In Rab3a-silenced cells, MLV Gag protein levels were significantly reduced. However, treatment with a lysosome inhibitor elevated the amount of Gag protein detected, showing that Rab3a suppresses lysosomal degradation of the Gag protein. Recently, we reported that Rab3a binds to CD63 to enhance lysosomal degradation of CD63 (8). In the present study, we also found that Rab3a binds to the MLV Gag protein and that this binding interferes with the interaction between Rab3a and CD63, which results in the inhibition of Rab3a-mediated lysosomal

279 degradation of CD63. SOCS1 binds and stabilizes the HIV-1 Gag protein (28); in SOCS1-silenced

280 cells, a lysosomal inhibitor enhanced the HIV-1 Gag protein level, showing that SOCS1 inhibits the

281 lysosomal degradation of the HIV-1 Gag protein. Rab3a functions in a similar manner in MLV

282 replication.

283 CD63 is required for efficient infection by released MLV particles, but not for virion production. CD63

284 silencing attenuates HIV-1 virion production (6-8), but it did not have this effect on MLV virion

285 production. A CD63 mutant containing amino acid substitutions at conserved cysteine residues

286 (CD63-TCS) consistently inhibited HIV-1 virion production; however, this effect was not observed in

287 MLV virion production (9). However, CD63-silencing or CD63-TCS expression decreased the

288 infectivity of released MLV particles. These results suggest that CD63 is required for efficient

289 infection by released MLV particles but not for MLV virion production.

290 Previous studies have shown that HIV-1 Vpu and Nef proteins decrease tetraspanin protein levels

291 (29,30). Conversely, the MLV Gag protein elevated CD63 levels. CD63 is required for efficient HIV-

292 1 particle production (6-8), but it inhibits the infectivity of released HIV-1 virions (20). CD63 has

293 advantageous functions in HIV-1 virion production and disadvantageous functions in HIV-1 entry into

294 host cells. HIV-1 should downregulate CD63 to suppress its disadvantageous role in viral entry.

295 Because CD63 expression is only moderately reduced by Vpu or Nef (29), the downregulation of

296 CD63 expression should have no effect on HIV-1 virion production. Conversely, CD63 expression did

297 not inhibit the infectivity of released MLV particles. CD63 has only advantageous functions in MLV

298 replication; it does not show disadvantages as observed in HIV-1. Therefore, MLV would benefit from

299 upregulating CD63 expression.

300 Rab3a RS-HA efficiently recovered MLV Gag protein levels in shRab3a-expressing cells, but

301 moderately increased transduction titers. Rab3a WT-HA reduced transduction titers, but did not reduce

302 Gag p30 levels in the virion fractions, suggesting that exogenous Rab3a expression inhibits the

303 infectivity of released MLV particles.

304 The inactive form of Rab3a enhanced MLV infectivity. Rab3a T36N-HA increased transduction titers,

305 but both WT-HA and Q81L-HA reduced them. These Rab3a proteins were detected in the virion

306 fractions in equal measure and did not affect MLV virion production. This data, taken together,

307 suggests that MLV particles containing the inactive form of Rab3a are more infectious than those

308 containing the active form. Rab3a WT-HA decreased transduction titers in a similar manner as Rab3a

309 Q81L-HA, suggesting that a large proportion of the Rab3a protein in 293T cells binds to GTP.

310 Rab3a is essential for lysosome exocytosis and plasma membrane repair (26,27). Rab3a-mediated

311 lysosome exocytosis may be involved in MLV virion production; however, SLO-induced plasma

312 membrane injury did not affect MLV vector production. Although the active GTP-binding form of

313 Rab3a induces lysosome transport to the plasma membrane (27), MLV virion production was

314 independent of the Rab3a activation state. Therefore, it is thought that Rab3a only stabilizes the MLV

315 Gag protein and does not mediate Gag protein transport. Further study is required for understanding

316 the underlying mechanism of MLV Gag protein stabilization by Rab3a.

317

Materials and Methods

Cells. Human 293T and TE671 cells were maintained in our laboratory over a long period of time. These cells were cultured in Dulbecco's-modified Eagle's medium with 8% fetal bovine serum and 1% penicillin-streptomycin. To construct Rab3a-silenced cells, a lentiviral vector encoding shRNA against Rab3a mRNA was inoculated into 293T or TE671 cells. The inoculated cells were selected using puromycin; the puromycin-resistant cell pool was used in this study. To construct an ecotropic MLV receptor (mCAT1)-expressing TE671 cells, TE671 cells were transfected with a plasmid encoding mCAT1 and a neomycin-resistant gene. Transfected cells were selected using geneticin (Promega), and the geneticin-resistant cell clones were isolated. To identify which cell clones

327 expressed mCAT1, cell clones were inoculated with the LacZ-encoding ecotropic MLV vector (31)

328 and transduction titers were measured. The most susceptible cell clone (TE671-mCAT1) was used in

329 this study.

330

331 **Plasmids.** A plasmid expressing C-terminally GFP-tagged CD63 was constructed in our previous

332 study (9). C-terminally HA-tagged Rab3a WT, T36N, Q81L, and RS expression plasmids were

333 constructed in another previous study (8). The lentiviral vector genome expression plasmid encoding

334 shRab3a was constructed in our laboratory. The target sequence of the shRab3a was GGACAAC

335 AUUAAUGUCAAG. The Env expression plasmid was also constructed in our laboratory (31). The

336 MLV Gag-Pol protein expression plasmid was purchased from TaKaRa.

337

338 **MLV vector.** To construct an amphotropic MLV vector, 293T cells were transfected with MLV Gag-

339 Pol, amphotropic Env, and LacZ-encoding MLV vector genome expression plasmids. Culture

340 supernatants from the transfected cells were inoculated into TE671 cells in the presence of polybrene.

341 Inoculated cells were cultured for 2 days and stained with X-Gal (Wako). The number of blue cells

342 was counted to estimate transduction titers. To analyze the effect of SLO on MLV vector production,

343 293T cells were treated with SLO (Bio Academia) for 10 min, 24 h after transfection; washed with

PBS; and cultured in fresh medium for 24 h. Culture supernatants from the treated cells were inoculated into TE671 cells.

Replication-competent MLV. TE671 cells were transfected with an ecotropic MLV receptor (mCAT1) and selected using geneticin. Geneticin-resistant cell clones were isolated. To determine which cell clones expressed mCAT1, the cells were inoculated with an ecotropic MLV vector encoding the LacZ marker gene, and transduction titers were measured. The most susceptible cell clone (TE671-mCAT1) was used for the following experiments. 293T cells were transfected with a plasmid encoding replication-competent Moloney MLV (21). Culture supernatant from the transfected cells was

353 inoculated into TE671-mCAT1 cells, and the inoculated cells were maintained for at least 2 weeks.

354 The culture supernatant of the cells was used in this study.

355

356 **Western immunoblotting.** Virion fractions were collected as follows. Culture supernatants were

357 centrifuged at 1,000 rpm for 10 min to remove cells and cell debris and were further centrifuged at

358 12,000 rpm for 4 h through 20% sucrose. The resulting pellets were used as virion fractions. Cell

359 lysates and virion fractions were subjected to SDS-PAGE (Bio-Rad), and the proteins were transferred

360 onto PVDF membranes (Millipore). When the membranes were treated with mouse anti-GFP (Nacalai

361 Tesque, Inc.), anti-HA antibodies (Covance), or anti-actin (Santa Cruz Biotechnology), they were also

362 treated with HRP-conjugated anti-mouse IgG antibodies (Bio-Rad). When the membranes were

363 treated with goat anti-MLV Gag p30 antiserum (ViroMed), they were also treated with HRP-

364 conjugated protein G (Bio-Rad). The antibody-bound proteins were visualized using ECL reagent

365 (Bio-Rad).

366 Because the endogenous Rab3a protein was not detected by direct Western blotting, Rab3a protein in

367 cell lysates was concentrated by immunoprecipitation. Rabbit anti-Rab3a antibodies (Santa Cruz

368 Biotechnology) and protein G-agarose beads (Sigma-Aldrich) were added to the lysates and these

369 lysates were then incubated at 4°C for 4 h. The precipitates were analyzed by Western immunoblotting

using anti-Rab3a and anti-rabbit IgG antibodies (GeneTex). This only detected native IgG; thus, the detection of denatured IgG present in the precipitates was avoided.

Immunoprecipitation. Appropriate antibodies were added to cell lysates and incubated at 4°C for 4 h. Then, anti-mouse IgG antibody- s or protein G-agarose beads (Sigma-Aldrich) were added and incubated at 4°C for a further 4 h. The beads were washed with lysate buffer 5 times; then, the sample buffer was added to the beads. The supernatants were analyzed by Western blotting.

Confocal microscopy. 293T cells were transfected with Rab3a WT-HA and MLV Gag-Pol expression plasmids. The transfected cells were permeabilized with methanol and treated with rabbit anti-Rab3a and goat anti-MLV Gag p30 antibodies and then with FITC-conjugated anti-rabbit and PE-conjugated anti-goat IgG antibodies. Finally, the treated cells were observed by confocal microscopy (Olympus).

Statistical analysis. Differences between two sets of data were analyzed using the Student's t-test; differences were considered to be significant at p values < 0.05.

386 **References**

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488
489
490

491 **Figure legends**

492 **Fig. 1 CD63 is incorporated into MLV particles**

493 293T cells were transfected with CD63-GFP expression plasmids together with pcDNA3.1 or

494 amphotropic MLV vector construction plasmids. Cell lysates (left panel) and virion fractions (right

495 panel) prepared from the transfected cells were analyzed by Western blotting.

496

497 **Fig. 2 Exogenous CD63 expression has no effect on MLV virion production and infectivity**

498 (A) 293T cells were transfected with amphotropic MLV construction plasmids together with empty

499 or CD63-GFP expression plasmids. Transduction titers of the culture supernatants from the transfected

500 cells were measured. Transduction titers of the empty plasmid-transfected cells were always set to 1.

501 Relative values of transduction titers of the empty plasmid-transfected cells are indicated. Mean values

502 are shown, and error bars show standard deviations. (B) Cell lysates and virion fractions prepared

503 from transfected cells were analyzed by Western blotting.

504

505 **Fig. 3 CD63 silencing inhibits infectivity of released MLV particles**

506 (A) Control and shCD63-expressing TE671 cells were transfected with amphotropic MLV vector

507 construction plasmids. Transduction titers of the culture supernatants from the transfected cells were

508 measured. Transduction titers of control cells were always set to 1. Relative values of control cell

509 transduction titers are indicated. Asterisks indicate statistically significant differences. (B) Cell lysates

510 and virion fractions from the transfected cells were analyzed by Western blotting. (C) Replication-

511 competent Moloney MLV-producing cells were transduced with the shCD63-expressing lentiviral

512 vector. Viral titers of culture supernatants from the empty or shCD63-transduced cells were measured.

513 (D) Cell lysates and virion fractions from the transduced cells were analyzed by Western blotting.

514

515 **Fig. 4 Rab3a silencing significantly reduces the MLV Gag protein level**

516 (A) Endogenous Rab3a levels in the empty or shRab3a-encoding lentiviral vector-transduced 293T

517 cells were analyzed. (B) Rab3a-silenced cells were transfected with amphotropic MLV vector

518 construction plasmids. Transduction titers of culture supernatants from the transfected cells were

519 measured. Relative values of transduction titers in the empty vector-transduced cells are indicated.

520 (C) Cell lysates and virion fractions prepared from the transfected cells were analyzed by Western

521 blotting using anti-MLV Gag p30 antibodies (upper panel). Relative p30 levels normalized by actin

522 are indicated (lower panel). (D) Replication-competent Moloney MLV-producing TE671 cells were

523 transduced with the empty or shRab3a-encoding lentiviral vector. MLV Gag p30 and Rab3a levels in

524 the transduced cells were analyzed by Western blotting. (E) Viral titers of the culture supernatants

525 from the transduced cells are indicated.

526

Fig. 5 Rab3a is required for MLV Gag protein expression

(A) Control and Rab3a-silenced 293T cells were transfected with amphotropic MLV vector construction plasmids together with pcDNA3.1 or Rab3a RS-HA expression plasmids. Transduction titers of the culture supernatants from the transfected cells were measured. Transduction titers of control cells transfected with pcDNA3.1 were always set to 1. Single asterisks indicate significant differences compared with titers of control cells transfected with pcDNA3.1. Double asterisks show significant differences compared with titers of Rab3a-silenced cells transfected with cDNA3.1. (B) Cell lysates prepared from the transfected cells were analyzed by Western blotting using anti-MLV Gag p30, anti-HA, and anti-actin antibodies.

536

537 **Fig. 6 The MLV Gag protein is degraded in the lysosomes of Rab3a-silenced cells**

538 (A) Control and Rab3a-silenced 293T cells were transfected with the MLV Gag-Pol expression

539 plasmid and treated with DMSO, concanamycin A (CMA), or MG-132. Cell lysates prepared from

540 the treated cells were analyzed by Western blotting using anti-MLV Gag p30 and anti-actin antibodies.

541 (B) Band intensities of MLV Gag p30 and actin proteins were measured using a densitometer, and p30

542 levels were normalized by actin levels. Normalized p30 levels of control cells treated with DMSO

543 were always set to 1. Single asterisks show significant differences compared with p30 levels of control

544 cells treated with DMSO. Double asterisks indicate significant differences compared with p30 levels

545 in the Rab3a-silenced cells treated with DMSO.

546

547 **Fig. 7 MLV inhibits Rab3a-mediated degradation of CD63**

548 (A) 293T cells were transfected with Rab3a WT-HA and CD63-GFP expression plasmids with or

549 without MLV Gag-Pol expression plasmids. Cell lysates prepared from transfected cells were

550 analyzed by Western blotting using anti-GFP, anti-HA, anti-MLV Gag p30, or anti-actin antibodies.

551 (B) Cell lysates prepared from replication-competent Moloney MLV-producing and uninfected

552 TE671-mCAT1 cells were analyzed by Western blotting using anti-MLV p30, anti-CD63, or anti-actin

553 antibodies (left panel). CD63 levels normalized by actin levels were calculated. The normalized CD63

554 levels in uninfected cells were always set to 1, and relative values are indicated (right panel). Asterisks

555 indicate significant differences.

556

557 **Fig. 8 The MLV Gag protein inhibits interaction between CD63 and Rab3a**

558 (A) 293T cells were transfected with Rab3a WT-HA, T36N-HA, or Q81L-HA expression plasmids

559 together with pcDNA3.1 or MLV Gag-Pol expression plasmids. Cell lysates from the transfected cells

560 were immunoprecipitated with anti-MLV p30 antibodies. The precipitates were analyzed by Western

561 blotting using anti-HA antibodies (upper panel). Direct Western blots using the anti-MLV p30, anti-

562 HA, or anti-actin antibodies are also shown in the lower panels. (B) 293T cells were transfected with

563 various combinations of pcDNA3.1, Rab3a WT-HA, CD63-GFP, and MLV Gag-Pol expression

564 plasmids. Cell lysates prepared from the transfected cells were immunoprecipitated using anti-GFP

565 antibodies. The precipitates were analyzed by Western blotting using anti-HA antibodies. Direct

566 Western blotting was performed using anti-HA, anti-GFP, anti-MLV p30, or anti-actin antibodies.

567

568 **Fig. 9 Active and inactive forms of Rab3a decrease and increase MLV vector infectivity,**

569 **respectively**

570 (A) 293T cells were transfected with amphotropic MLV vector construction plasmids together with

571 pcDNA3.1, Rab3a WT-HA, T36N-HA, or Q81L-HA expression plasmids. Culture supernatants from

572 transfected cells were inoculated into TE671 cells and transduction titers were measured. Transduction

573 titers of pcDNA3.1-transfected cells were always set to 1. Asterisks indicate significant differences

574 compared with control cell titers. (B) Cell lysates and virion pellets from the transfected cells were

575 analyzed by Western immunoblotting. (C) p30 levels in virion pellets normalized by p30 levels in cell

576 lysates were calculated. Normalized p30 levels in virion pellets from the pcDNA3.1-transfected cells

577 were always set to 1, and relative values are indicated.

578

579 **Fig. 10 Mouse Rab3a stabilizes the MLV Gag protein**

580 (A) 293T cells were transfected with mRab3a RS-HA expression plasmids together with pcDNA3.1

581 or MLV Gag-Pol expression plasmids. The MLV Gag protein was precipitated by anti-p30 antibodies.

582 The precipitates were analyzed by Western immunoblotting using anti-HA antibodies (upper panel).

583 Cell lysates from the transfected cells were directly analyzed by Western blotting (lower panel). (B)

584 Control and Rab3a-silenced 293T cells were transfected with VSV-pseudotyped MLV vector

585 construction plasmids together with pcDNA3.1 or mRab3a RS-HA expression plasmids. Culture

586 supernatants from the transfected cells were inoculated into 293T cells and transduction titers were

587 measured. Transduction titers of control cells were always set to 1. Single asterisks indicate significant

588 differences compared with control cell titers. Double asterisks show significant differences between

589 the two groups indicated. (C) Cell lysates from the transfected cells were analyzed by Western

590 immunoblotting.

591

592 **Supplementary Fig. 1 Rab3a is co-localized with the MLV Gag protein**

593 293T cells were transfected with Rab3a WT-HA and MLV Gag-Pol expression plasmids. The

594 transfected cells were treated with rabbit anti-Rab3a and goat anti-MLV p30 antibodies. The cells

595 were then treated with FITC-conjugated anti-rabbit IgG and PE-conjugated anti-goat IgG antibodies.

596 (A) Several cells were observed in the microscopic field. (B) Details of a single cell.

597

598 **Supplementary Fig. 2 Amino acid sequences of human and mouse Rab3a**

599 Bars indicate identical amino acids.

600

601 **Supplementary Fig. 3 SLO does not affect MLV virion production**

602 (A) 293T cells were transfected with amphotropic MLV vector construction plasmids and treated

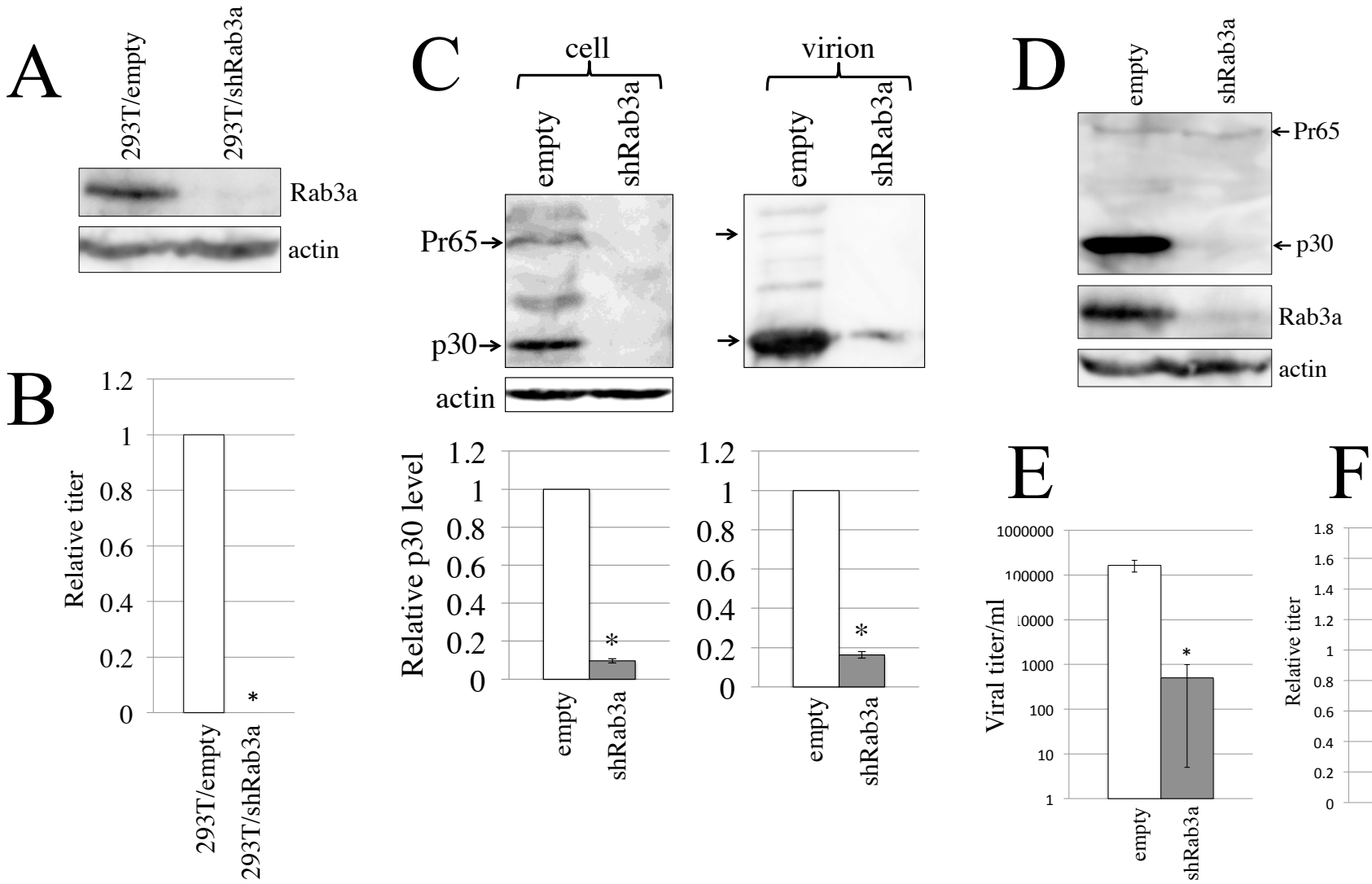
603 with SLO for 10 min. Western immunoblotting of cell lysates and virion fractions was performed

604 using anti-p30 and anti-actin antibodies. (B) Culture supernatants from SLO-treated cells were

605 inoculated into 293T cells and transduction titers were measured. Transduction titers of control cells

606 were always set to 1. Asterisks indicate significant differences compared with untreated cell titers.

Fig.1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293T/shRab3a
Rab3a
actin
B
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0.8
0.6
0.4
0.2
0
*
293T/empty
293T/shRab3a
C
cell
virion
empty
shRab3a
Pr65
p30
actin
Relative p30 level
D
Pr65
p30
Rab3a
actin
E
Viral titer/ml
1000000
100000
10000
1000
100
10
1
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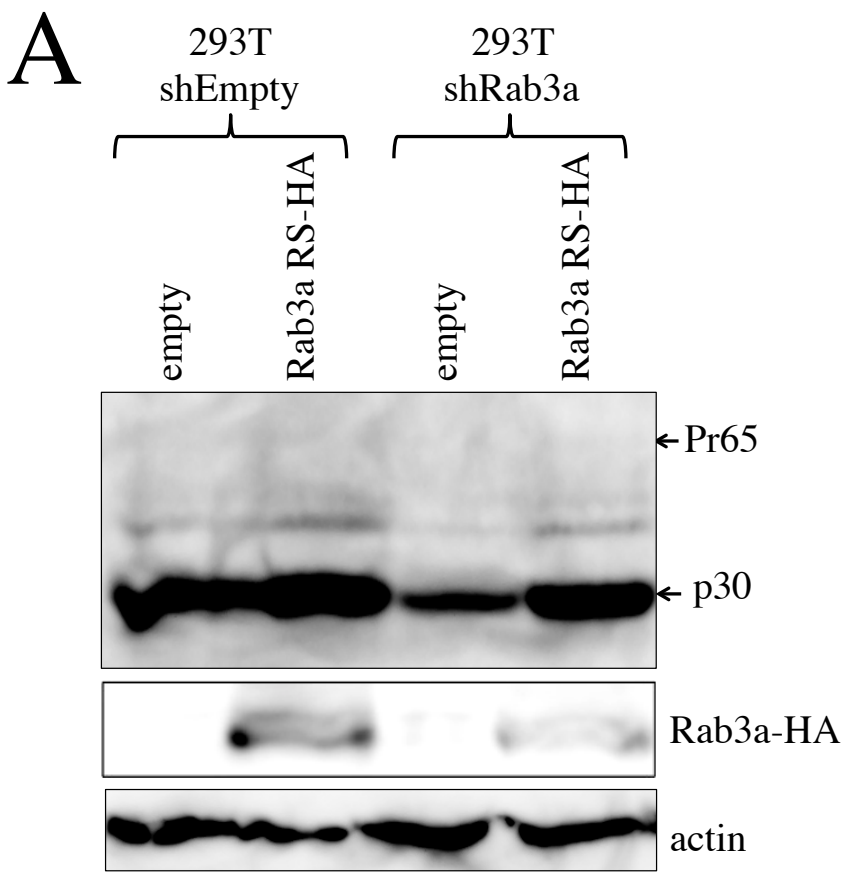
Fig.2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p30
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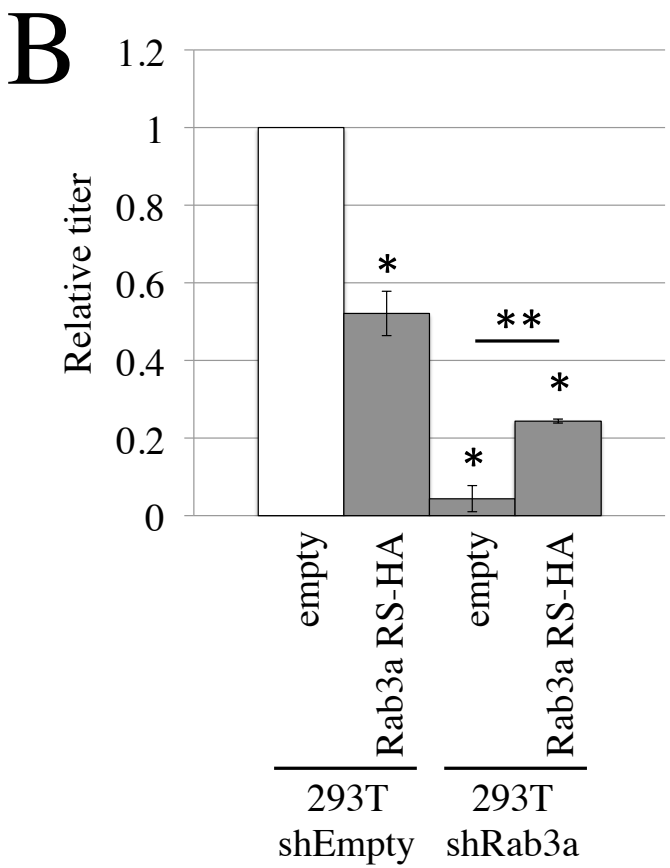
actin


B
Relative titer
1.2
1
0.8
0.6
0.4
0.2
0
*
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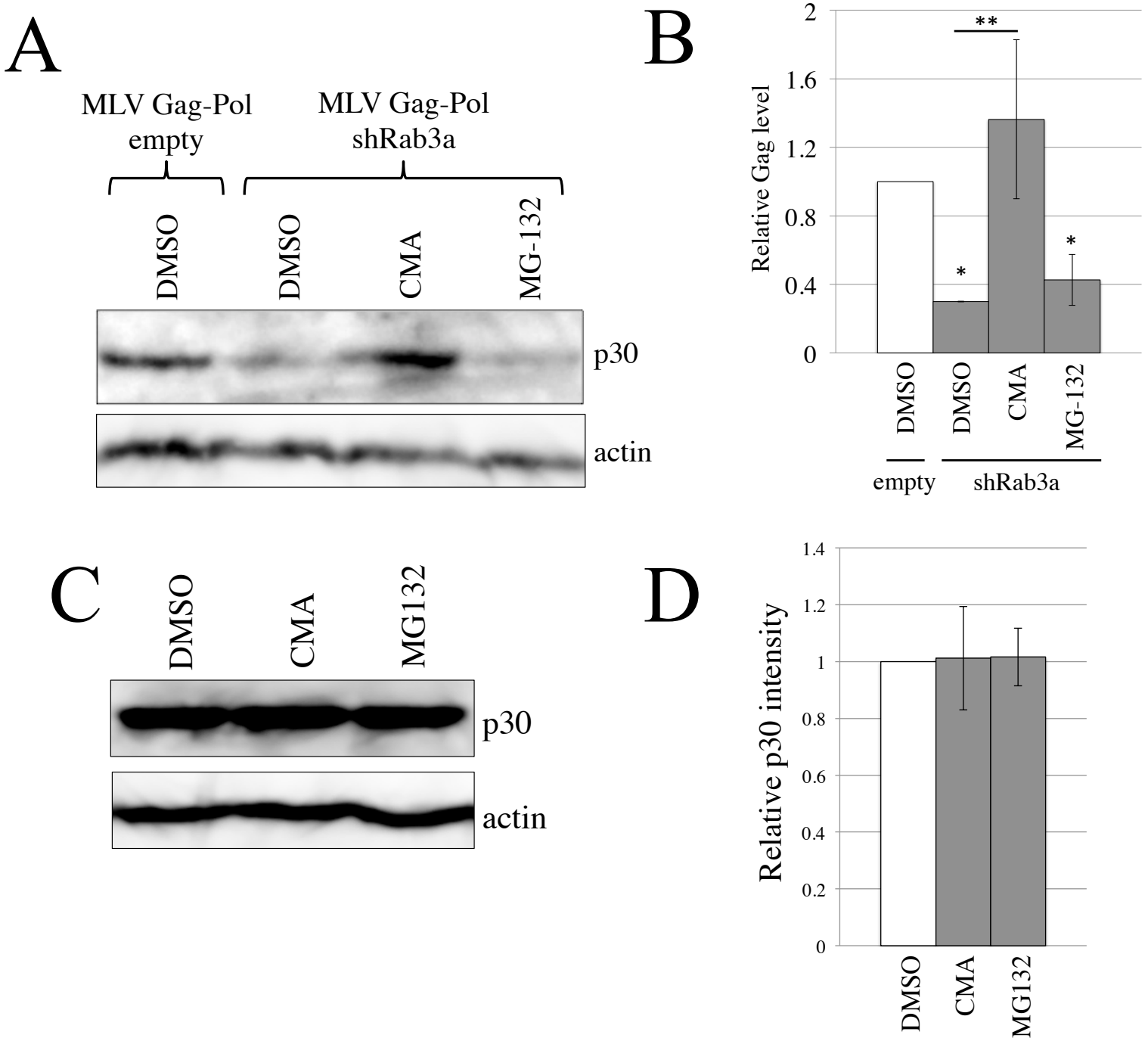
293T
shEmpty
293T
shRab3a

Fig.3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MLV Gag-Pol
shRab3a
DMSO
DMSO
CMA
MG-132
p30
actin
B
Relative Gag level
2
1.6
1.2
0.8
0.4
0
**
*
*
DMSO
DMSO
CMA
MG-132
empty
shRab3a
C
DMSO
CMA
MG132
p30
actin
D
Relative p30 intensity
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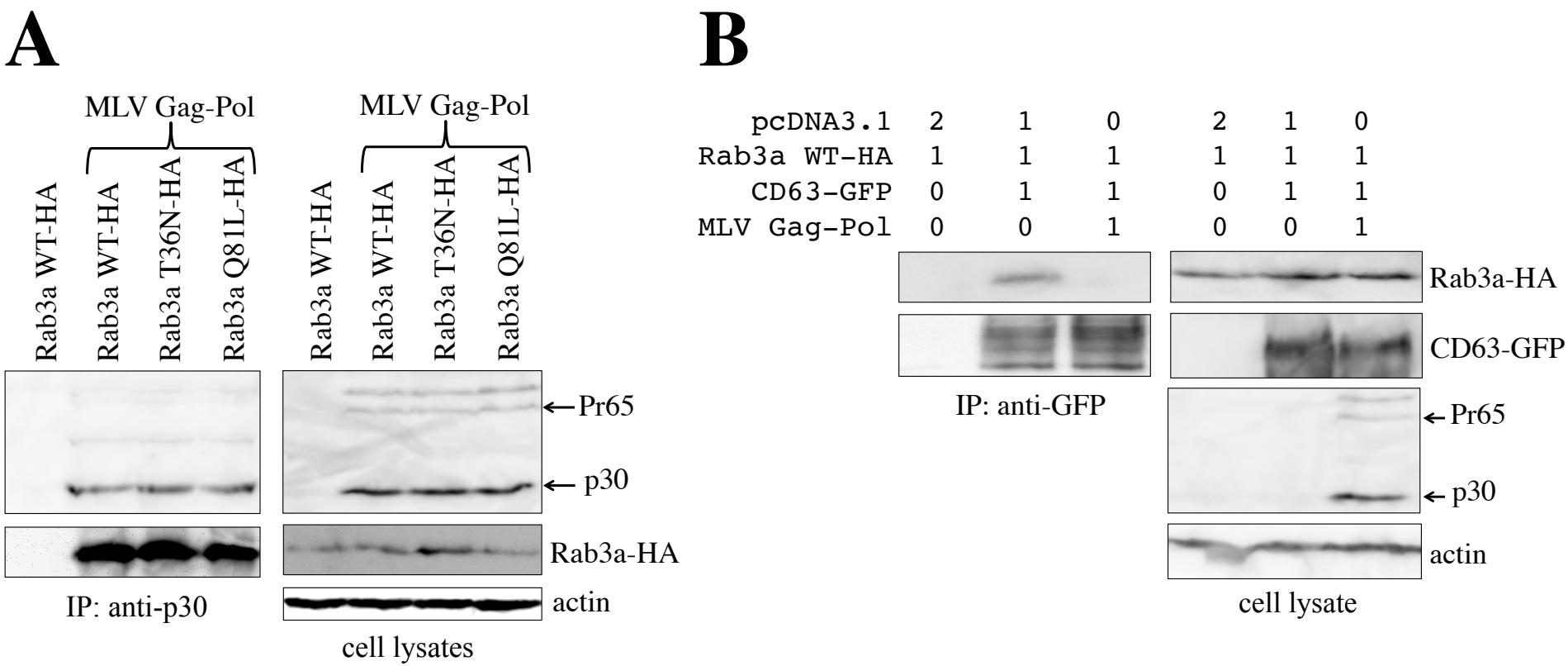
1.2
1
0.8
0.6
0.4
0.2
0
DMSO
CMA
MG132

Fig.4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MLV Gag-Pol
MLV Gag-Pol
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Rab3a WT-HA
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Rab3a WT-HA
Rab3a T36N-HA
Rab3a Q81L-HA
←Pr65
← p30
Rab3a-HA
actin
IP: anti-p30
cell lysates
B
pcDNA3.1 2 1 0 2 1 0
Rab3a WT-HA 1 1 1 1 1 1
CD63-GFP 0 1 1 0 1 1
MLV Gag-Pol 0 0 1 0 0 1
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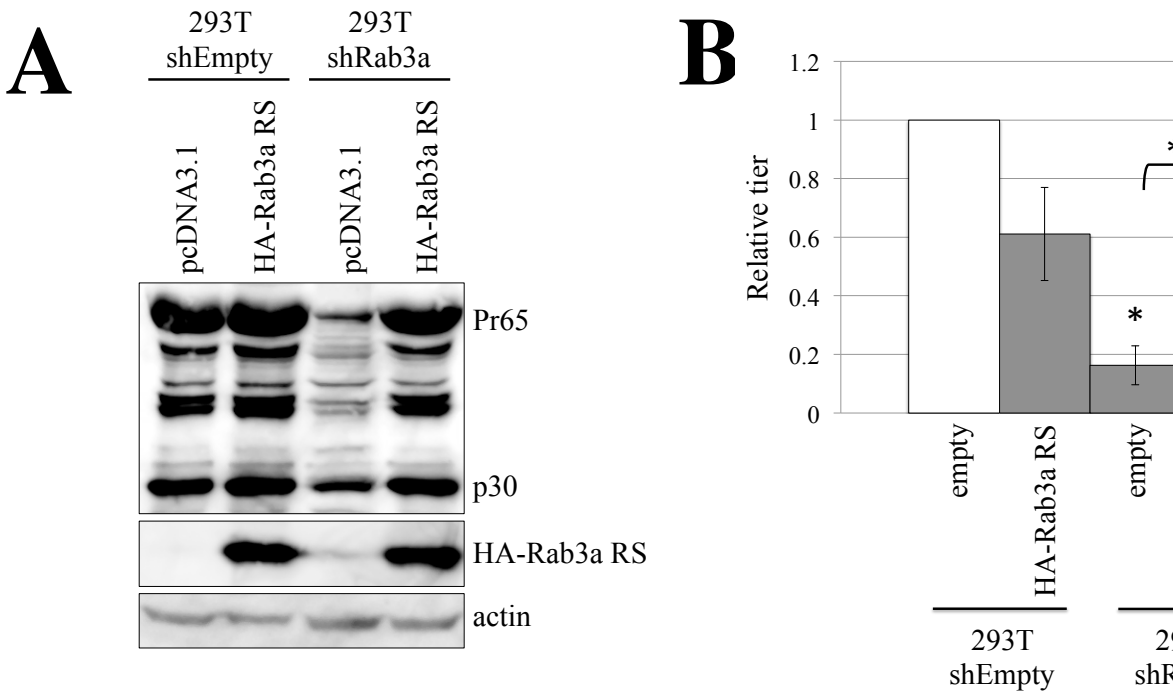
CD63-GFP
IP: anti-GFP
← Pr65
← p30
actin
cell lysate

Fig.5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293T shRab3a
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HA-Rab3a RS
pcDNA3.1
HA-Rab3a RS
Pr65
p30
HA-Rab3a RS
actin
B
Relative tier
1.2
1
0.8
0.6
0.4
0.2
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*
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empty
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empty
HA-Rab3a RS
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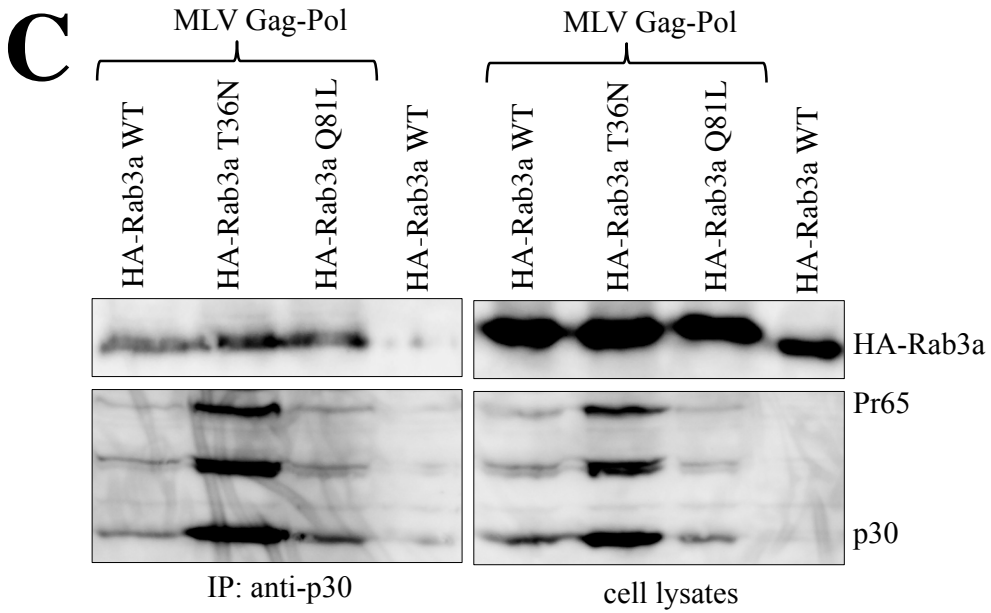
293T shRab3a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MLV Gag-Pol
MLV Gag-Pol
HA-Rab3a WT
HA-Rab3a T36N
HA-Rab3a Q81L
HA-Rab3a WT
HA-Rab3a WT
HA-Rab3a T36N
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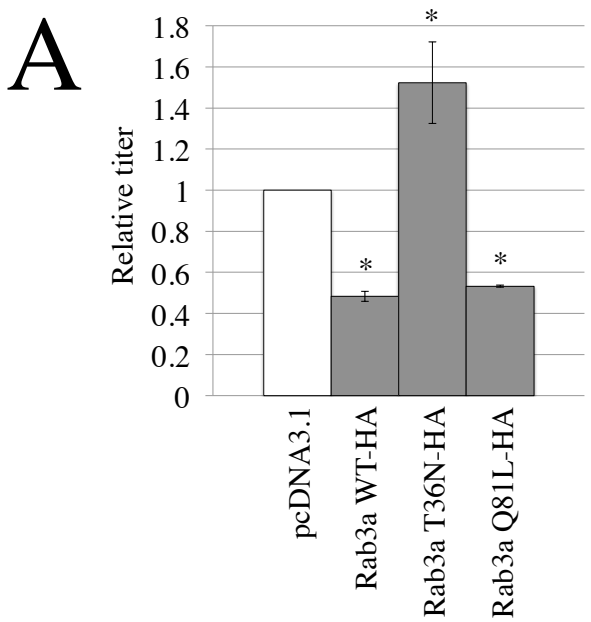
HA-Rab3a WT
HA-Rab3a
Pr65
p30
IP: anti-p30
cell lysates

Fig.6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Relative titer
1.8
1.6
1.4
1.2
1
0.8
0.6
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*
*
*
pcDNA3.1
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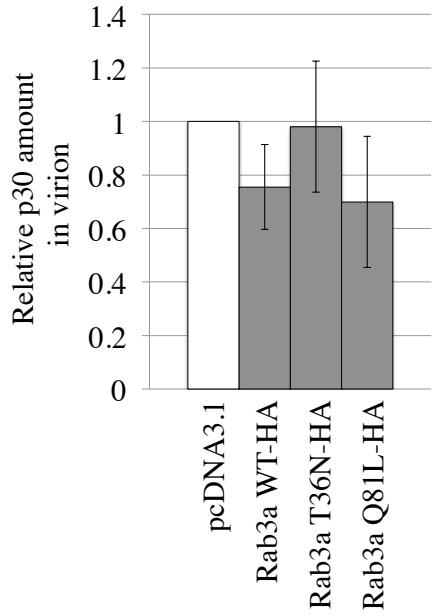
Rab3a T36N-HA
Rab3a Q81L-HA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Relative p30 amount in virion
1.4
1.2
1
0.8
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Rab3a T36N-HA
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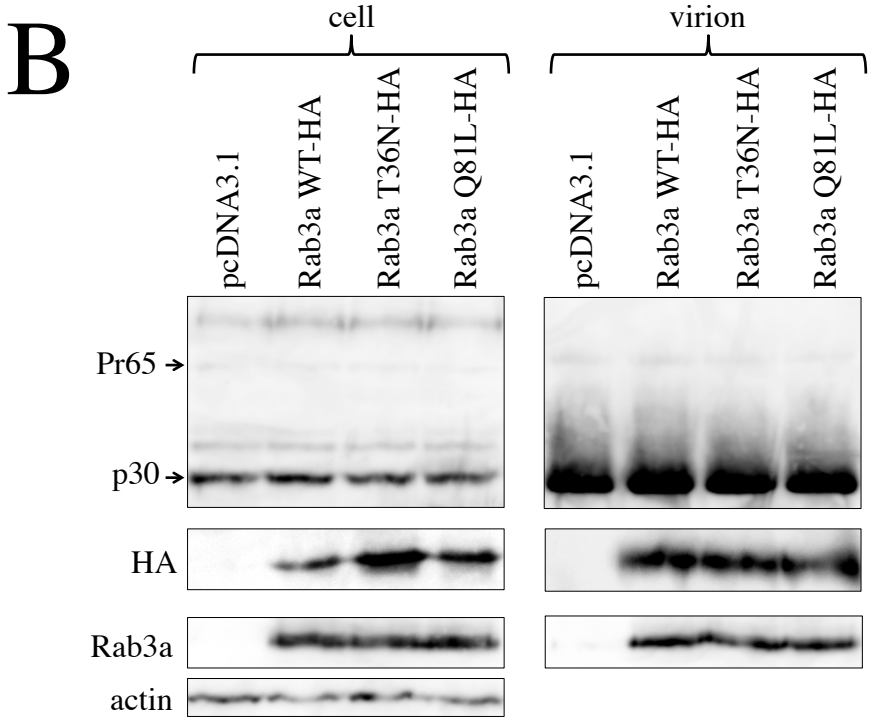
B
cell
virion
pcDNA3.1
Rab3a WT-HA
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Pr65
p30
HA
Rab3a
actin

Fig.7

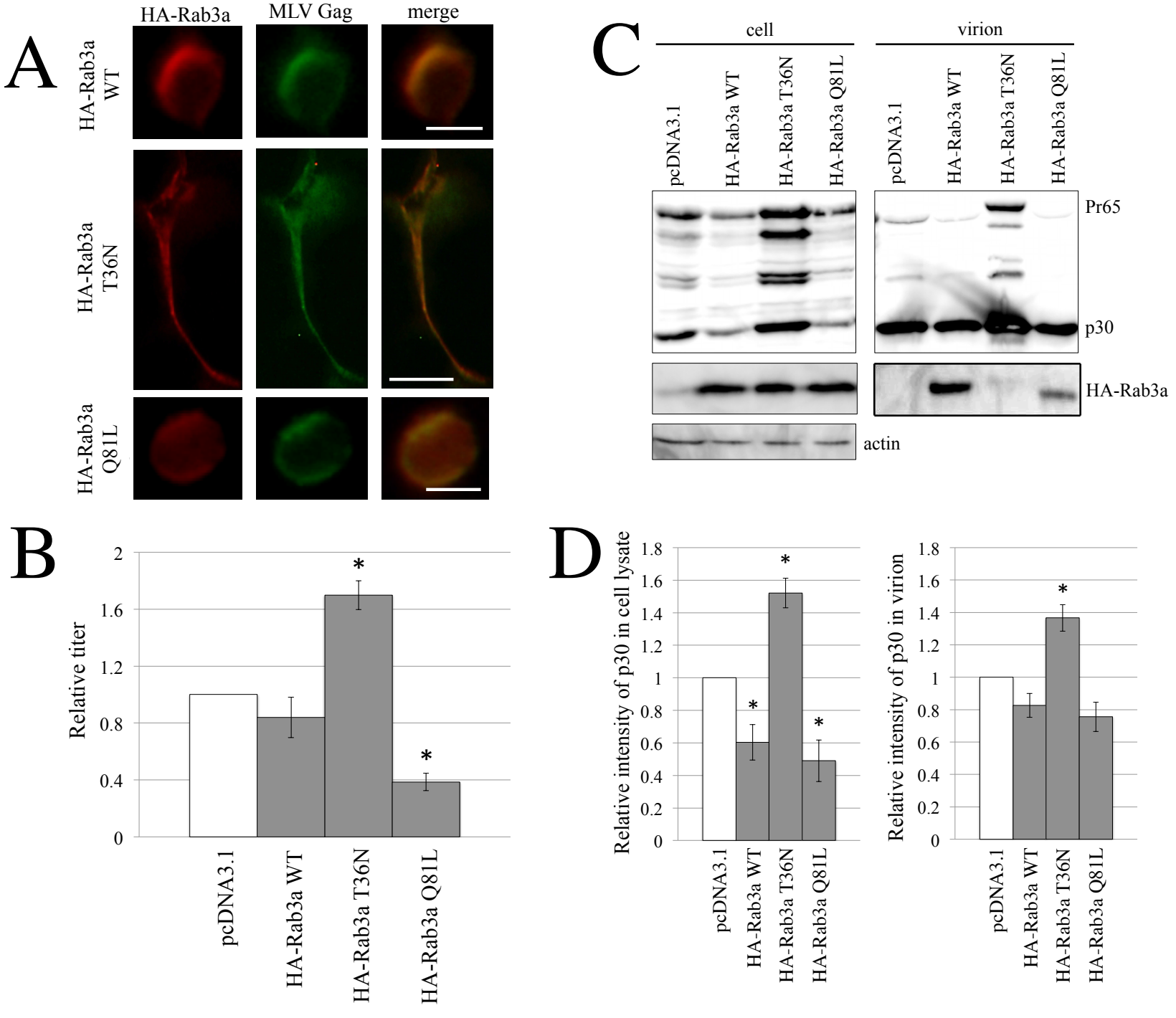

A
HA-Rab3a
MLV Gag
merge
HA-Rab3a WT
HA-Rab3a T36N
HA-Rab3a Q81L
C
cell
virion
pcDNA3.1
HA-Rab3a WT
HA-Rab3a T36N
HA-Rab3a Q81L
Pr65
p30
HA-Rab3a
actin
B
Relative titer
2
1.6
1.2
0.8
0.4
0
*
pcDNA3.1
HA-Rab3a WT
HA-Rab3a T36N
HA-Rab3a Q81L
D
Relative intensity of p30 in cell lysate
Relative intensity of p30 in virion
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*

Fig.8

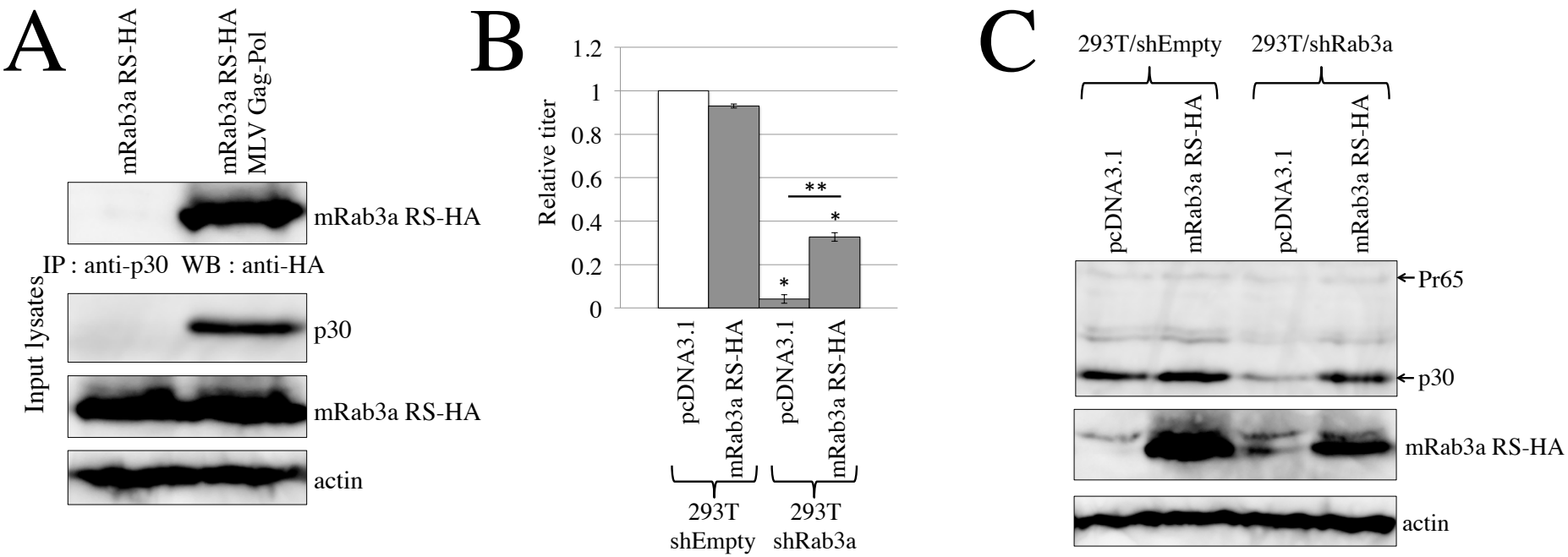

A
mRab3a RS-HA
mRab3a RS-HA
MLV Gag-Pol
mRab3a RS-HA
IP : anti-p30 WB : anti-HA
Input lysates
p30
mRab3a RS-HA
actin
B
Relative titer
1.2
1
0.8
0.6
0.4
0.2
0
**
*
*
pcDNA3.1
mRab3a RS-HA
pcDNA3.1
mRab3a RS-HA
293T shEmpty
293T shRab3a
C
293T/shEmpty
293T/shRab3a
pcDNA3.1
mRab3a RS-HA
pcDNA3.1
mRab3a RS-HA
Pr65
p30
mRab3a RS-HA
actin

Fig.9

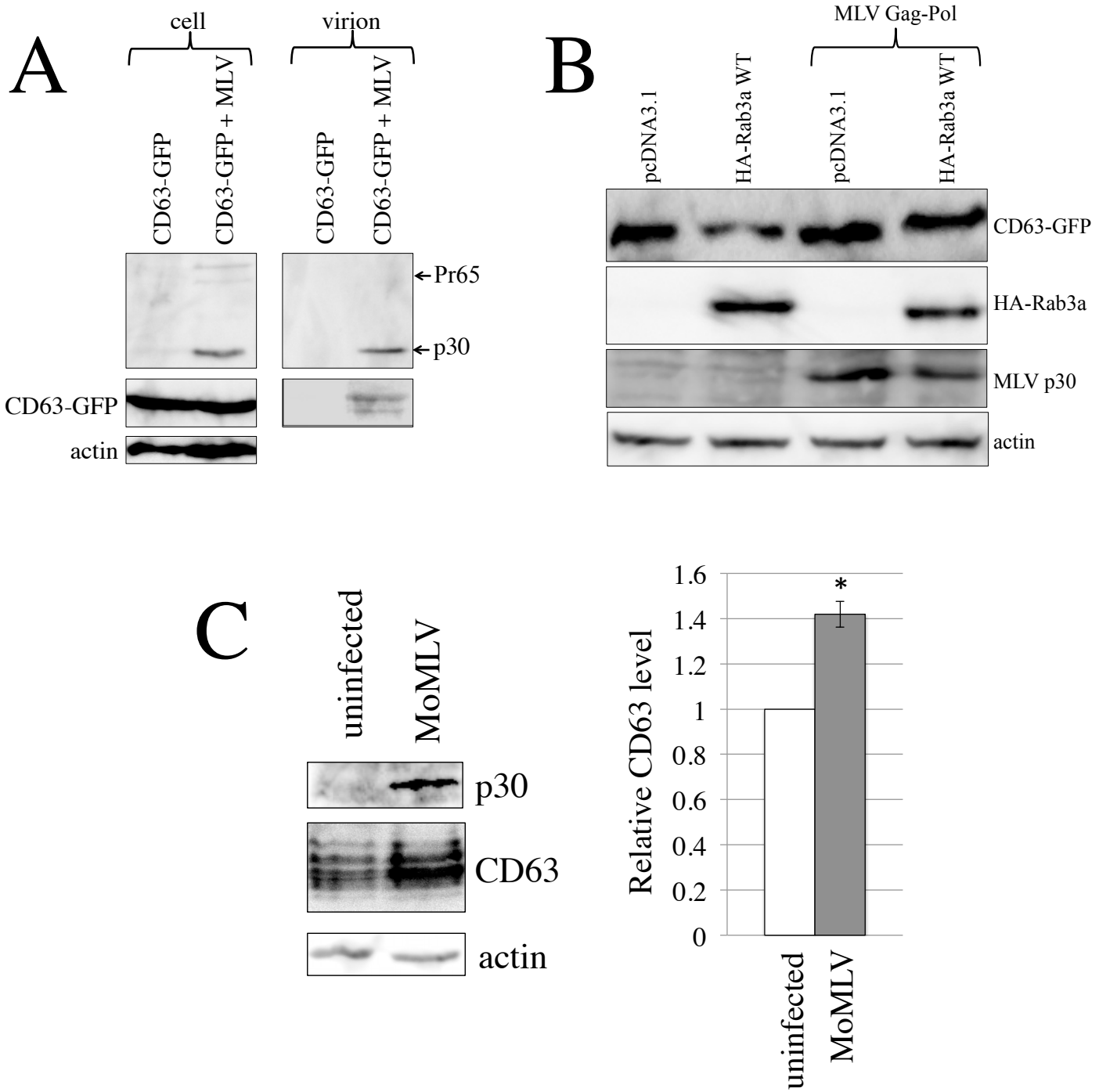

A
cell
virion
CD63-GFP
CD63-GFP + MLV
CD63-GFP
CD63-GFP + MLV
Pr65
p30
CD63-GFP
actin
B
MLV Gag-Pol
pcDNA3.1
HA-Rab3a WT
pcDNA3.1
HA-Rab3a WT
CD63-GFP
HA-Rab3a
MLV p30
actin
C
uninfected
MoMLV
p30
CD63
actin
Relative CD63 level
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
uninfected
MoMLV

Fig.10

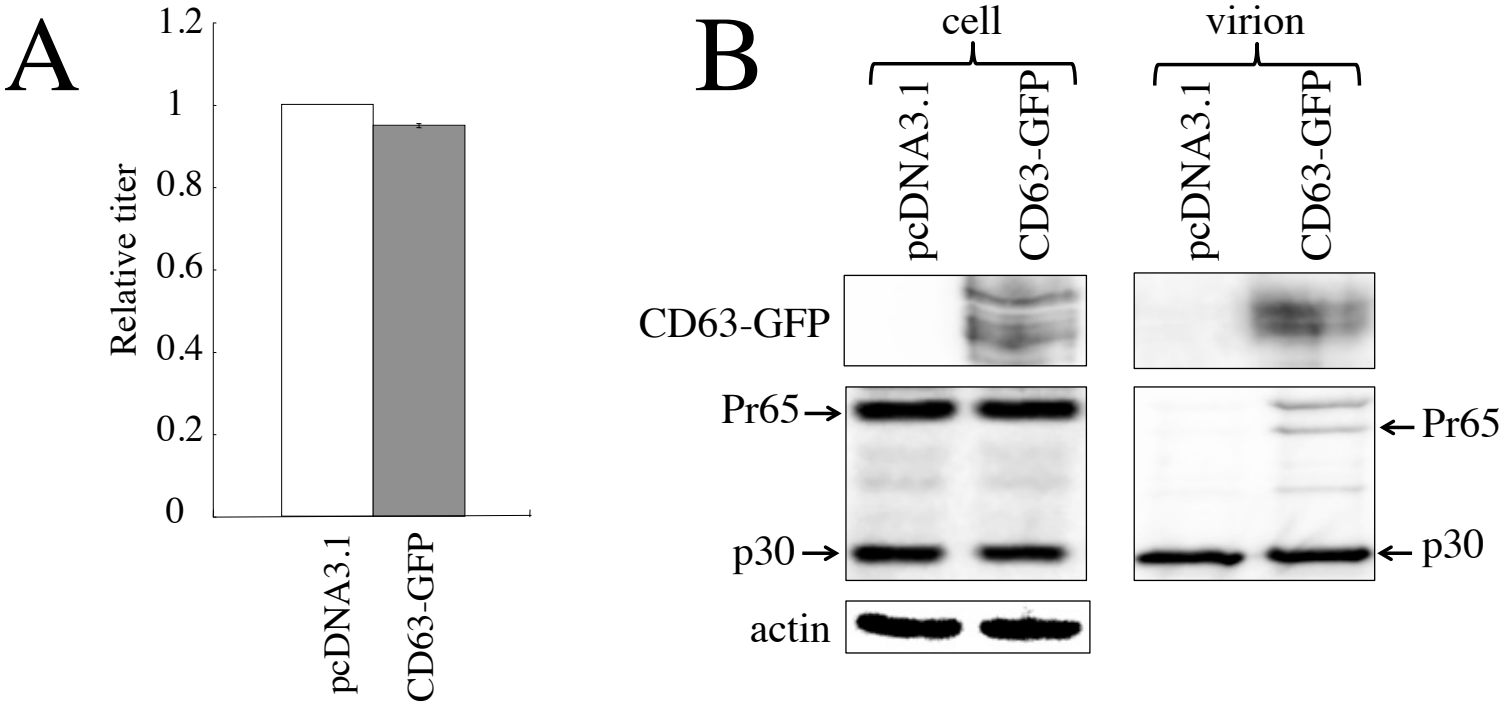

A
1.2
1
0.8
0.6
0.4
0.2
0
Relative titer
pcDNA3.1
CD63-GFP
B
cell
virion
pcDNA3.1
CD63-GFP
pcDNA3.1
CD63-GFP
CD63-GFP
Pr65→
p30→
actin
←Pr65
←p30

Fig.11

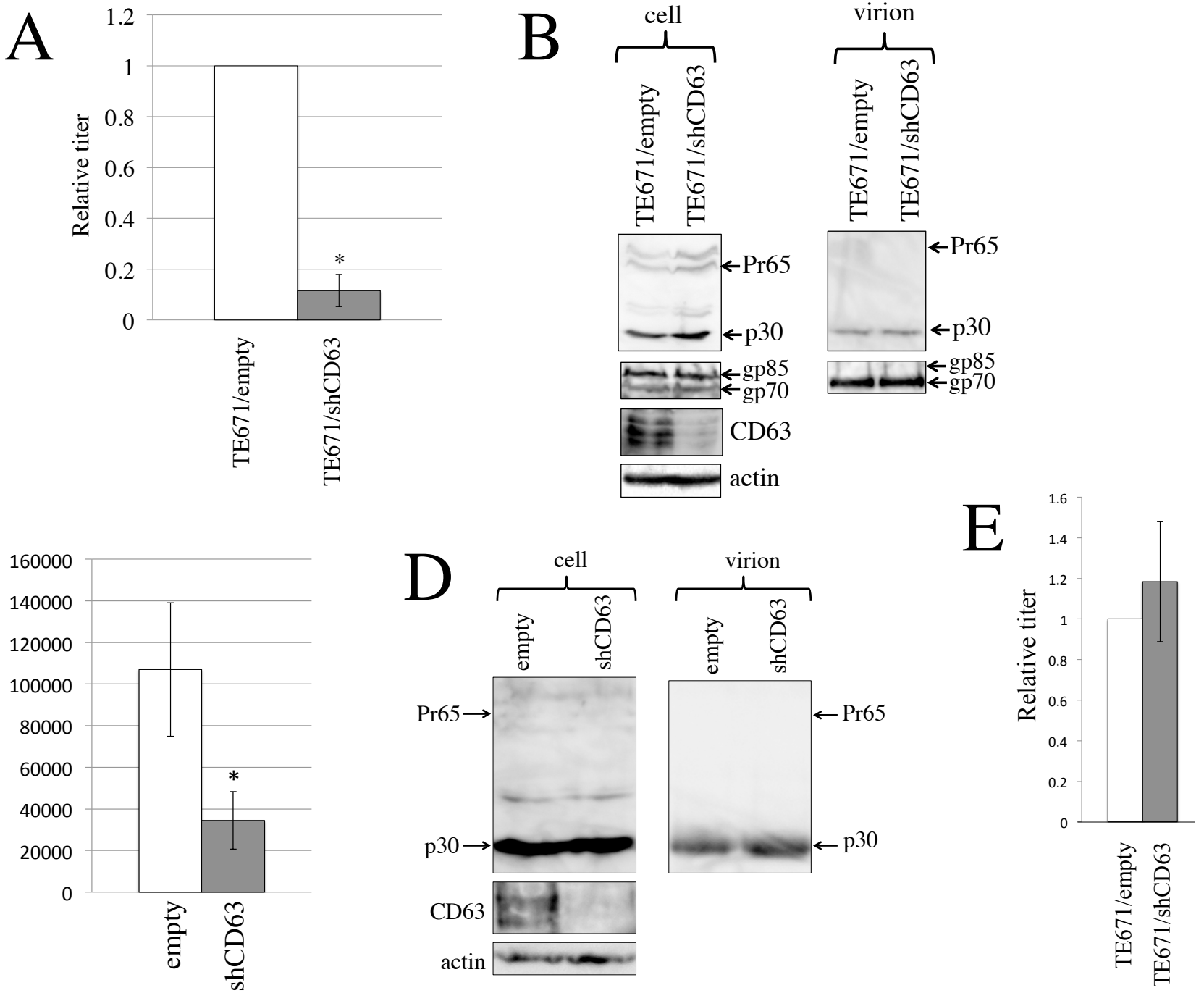

A
Relative titer
1.2
1
0.8
0.6
0.4
0.2
0
*
TE671/empty
TE671/shCD63
B
cell
virion
TE671/empty
TE671/shCD63
TE671/empty
TE671/shCD63
Pr65
p30
gp85
gp70
CD63
actin
Pr65
p30
gp85
gp70
C
viral titer
160000
140000
120000
100000
80000
60000
40000
20000
0
*
empty
shCD63
D
cell
virion
empty
shCD63
empty
shCD63
Pr65
p30
CD63
actin
Pr65
p30
E
Relative titer
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
TE671/empty
TE671/shCD63

Fig. 12

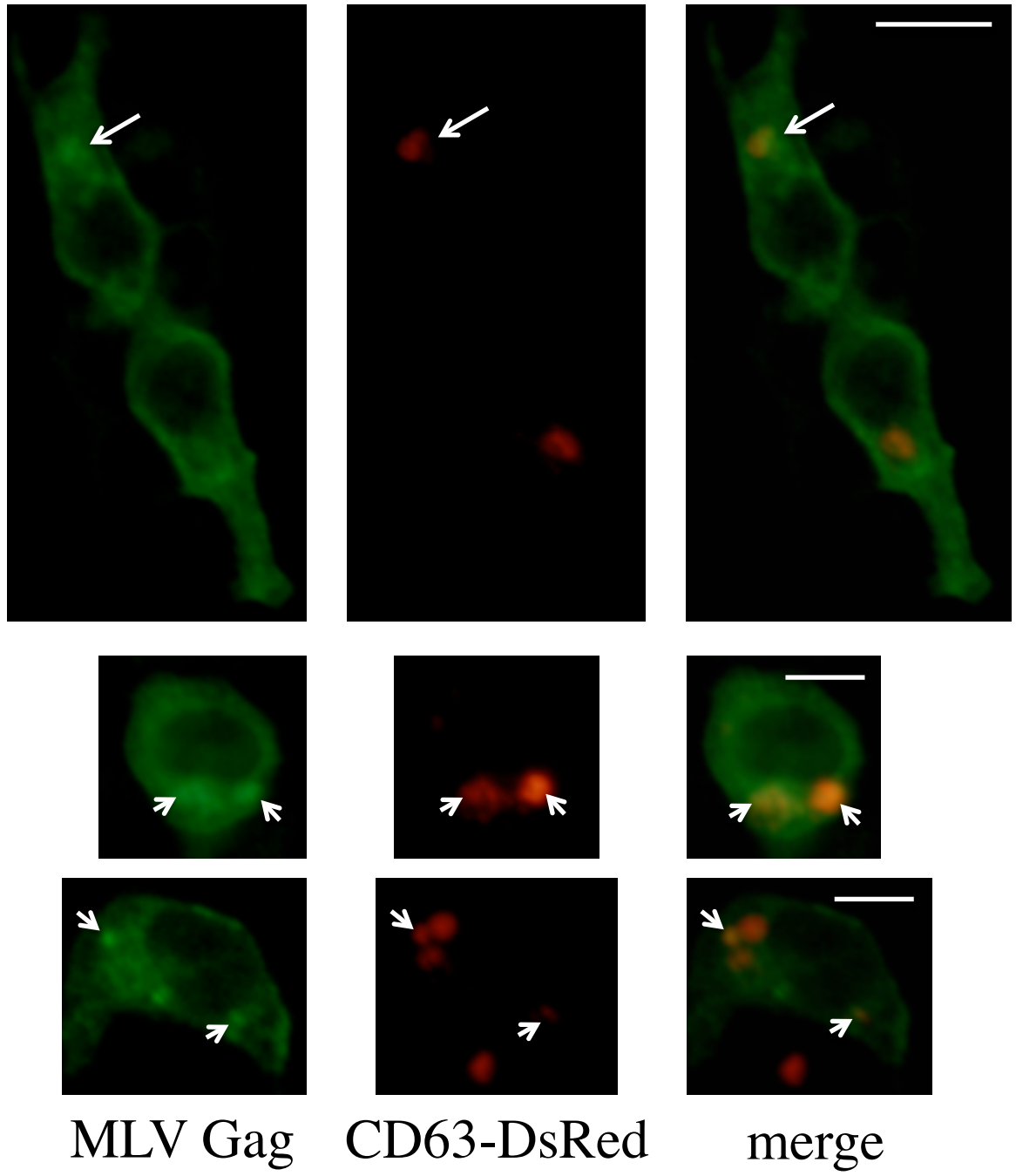
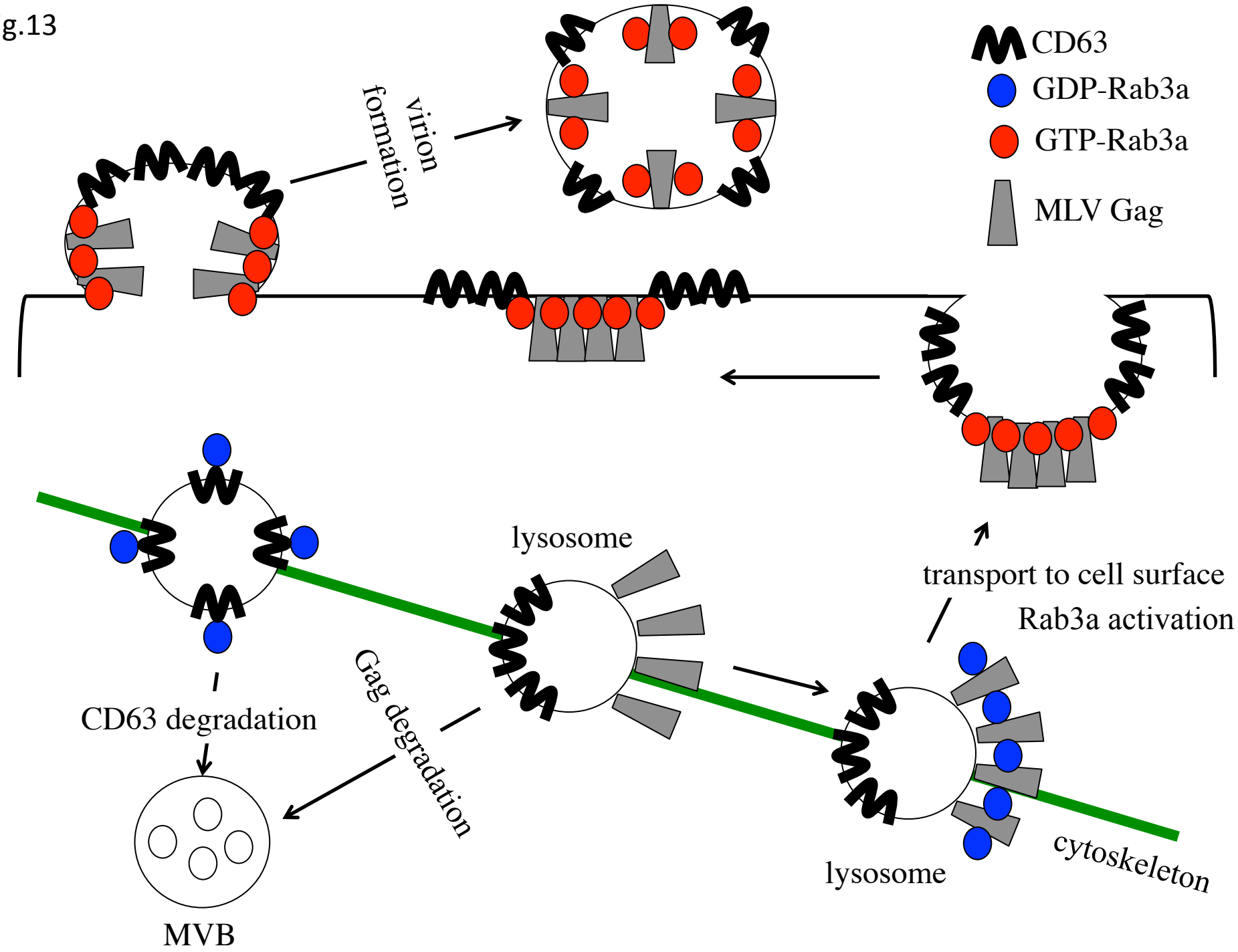


Fig.13


CD63
GDP-Rab3a
GTP-Rab3a
MLV Gag
virion formation
transport to cell surface
Rab3a activation
lysosome
CD63 degradation
Gag degradation
MVB
lysosome
cytoskeleton

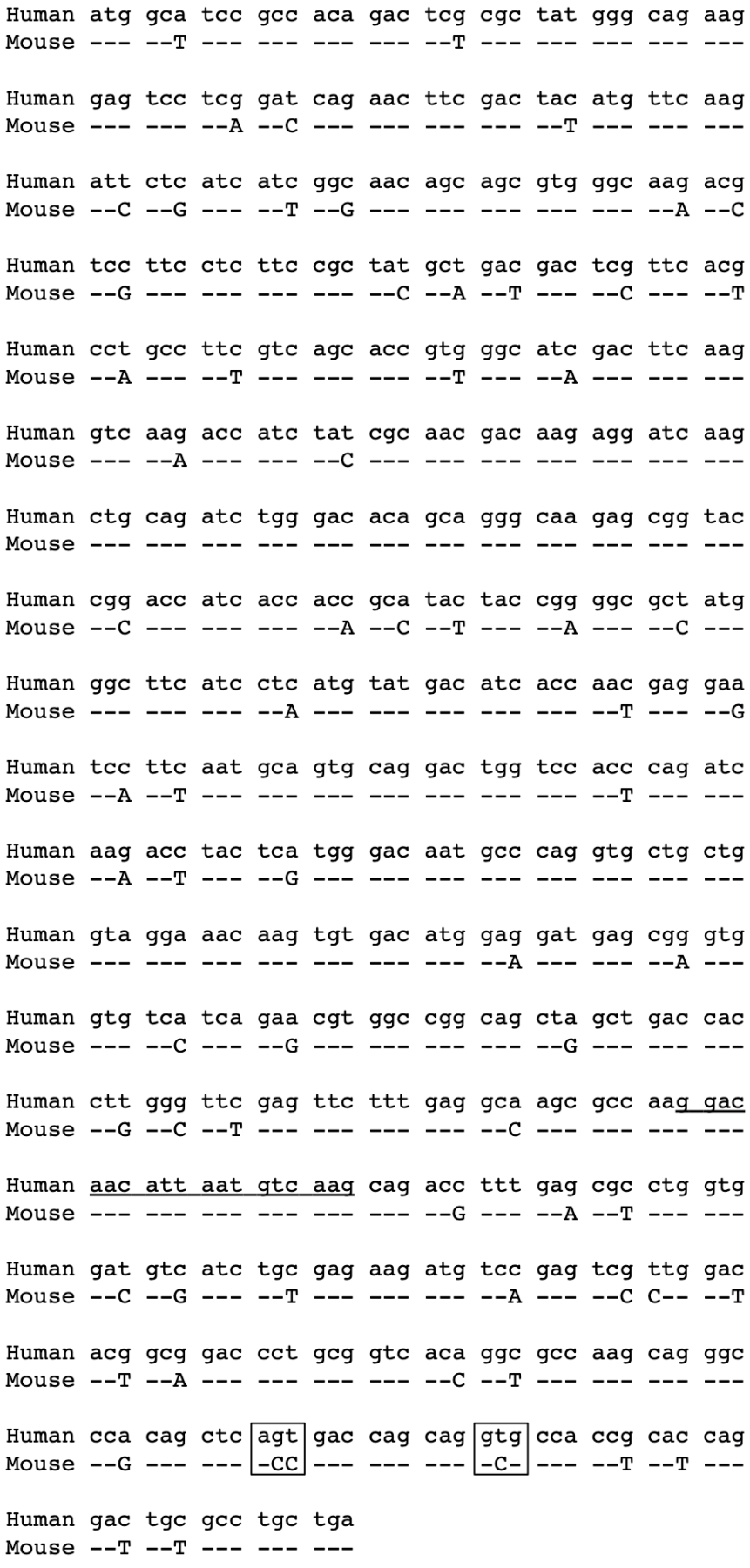

Human atg gca tcc gcc aca gac tcg cgc tat ggg cag aag
Mouse --- --T --- --- --- --- --T --- --- --- --- ---
Human gag tcc tcg gat cag aac ttc gac tac atg ttc aag
Mouse --- --- --A --C --- --- --- --- --T --- --- ---
Human att ctc atc atc ggc aac agc agc gtg ggc aag acg
Mouse --C --G --- --T --G --- --- --- --- --- --A --C
Human tcc ttc ctc ttc cgc tat gct gac gac tcg ttc acg
Mouse --G --- --- --- --- --C --A --T --- --C --- --T
Human cct gcc ttc gtc agc acc gtg ggc atc gac ttc aag
Mouse --A --- --T --- --- --- --T --- --A --- --- ---
Human gtc aag acc atc tat cgc aac gac aag agg atc aag
Mouse --- --A --- --- --C --- --- --- --- --- --- ---
Human ctg cag atc tgg gac aca gca ggg caa gag cgg tac
Mouse --- --- --- --- --- --- --- --- --- --- --- ---
Human cgg acc atc acc acc gca tac tac cgg ggc gct atg
Mouse --C --- --- --- --A --C --T --- --A --- --C ---
Human ggc ttc atc ctc atg tat gac atc acc aac gag gaa
Mouse --- --- --- --A --- --- --- --- --- --T --- --G
Human tcc ttc aat gca gtg cag gac tgg tcc acc cag atc
Mouse --A --T --- --- --- --- --- --- --- --T --- ---
Human aag acc tac tca tgg gac aat gcc cag gtg ctg ctg
Mouse --A --T --- --G --- --- --- --- --- --- --- ---
Human gta gga aac aag tgt gac atg gag gat gag cgg gtg
Mouse --- --- --- --- --- --- --- --A --- --- --A ---
Human gtg tca tca gaa cgt ggc cgg cag cta gct gac cac
Mouse --- --C --- --G --- --- --- --- --G --- --- ---
Human ctt ggg ttc gag ttc ttt gag gca agc gcc aag gac
Mouse --G --C --T --- --- --- --- --C --- --- --- ---
Human aac att aat gtc aag cag acc ttt gag cgc ctg gtg
Mouse --- --- --- --- --- --- --G --- --A --T --- ---
Human gat gtc atc tgc gag aag atg tcc gag tcg ttg gac
Mouse --C --G --- --T --- --- --- --A --- --C C-- --T
Human acg gcg gac cct gcg gtc aca ggc gcc aag cag ggc
Mouse --T --A --- --- --- --- --C --T --- --- --- ---
Human cca cag ctc agt gac cag cag gtg cca ccg cac cag
Mouse --G --- --- -CC --- --- --- -C- --- --T --T ---
Human gac tgc gcc tgc tga
Mouse --T --T --- --- ---

Fig. S2

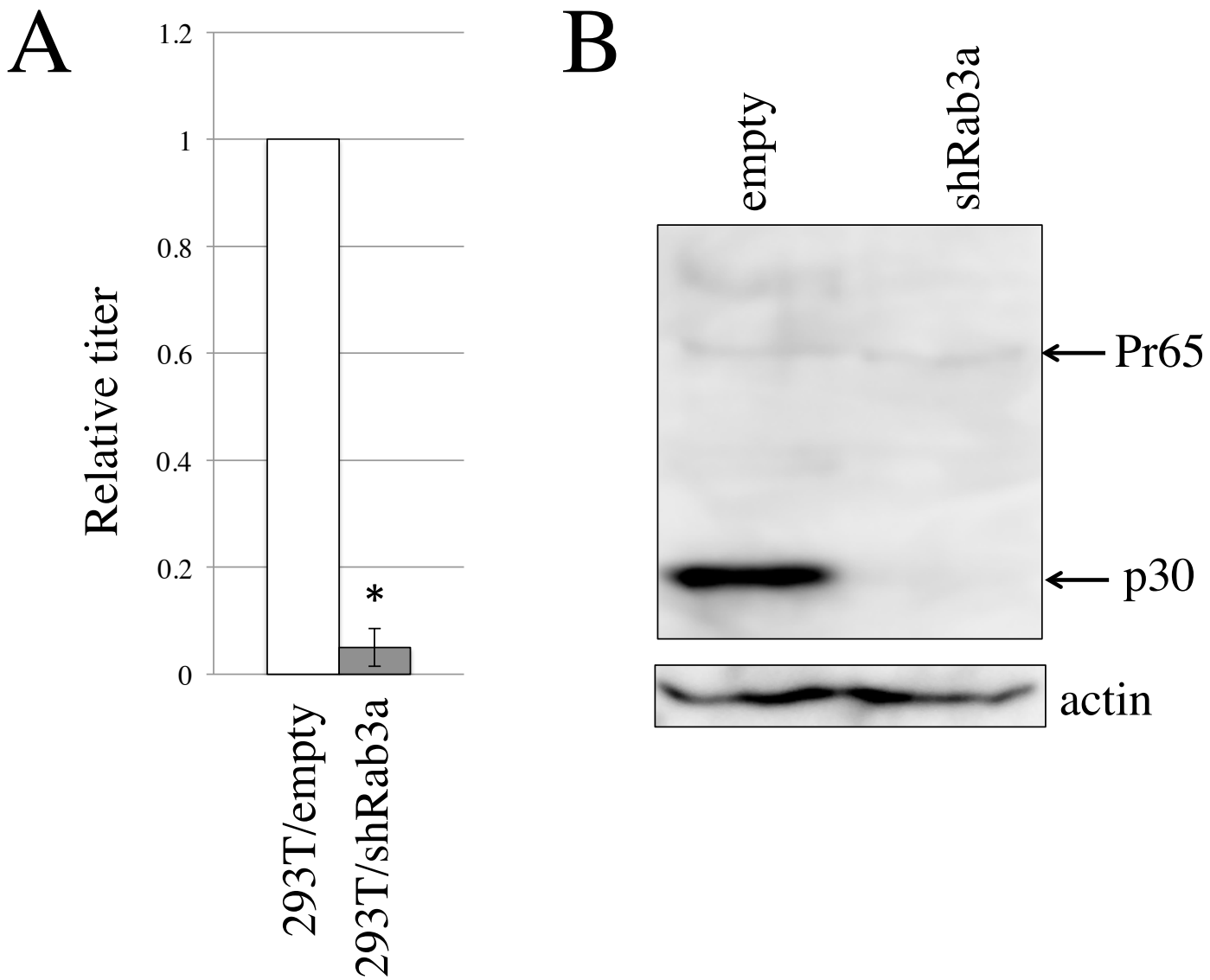

A
1.2
1
0.8
0.6
0.4
0.2
0
Relative titer
*
293T/empty
293T/shRab3a
B
empty
shRab3a
Pr65
p30
actin

Fig. S3

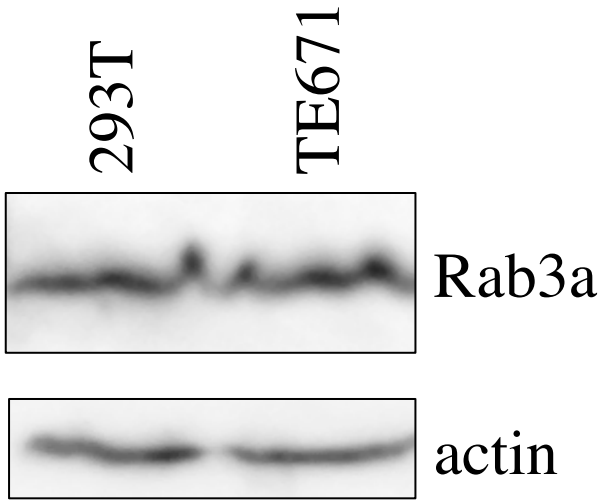


Fig. S4

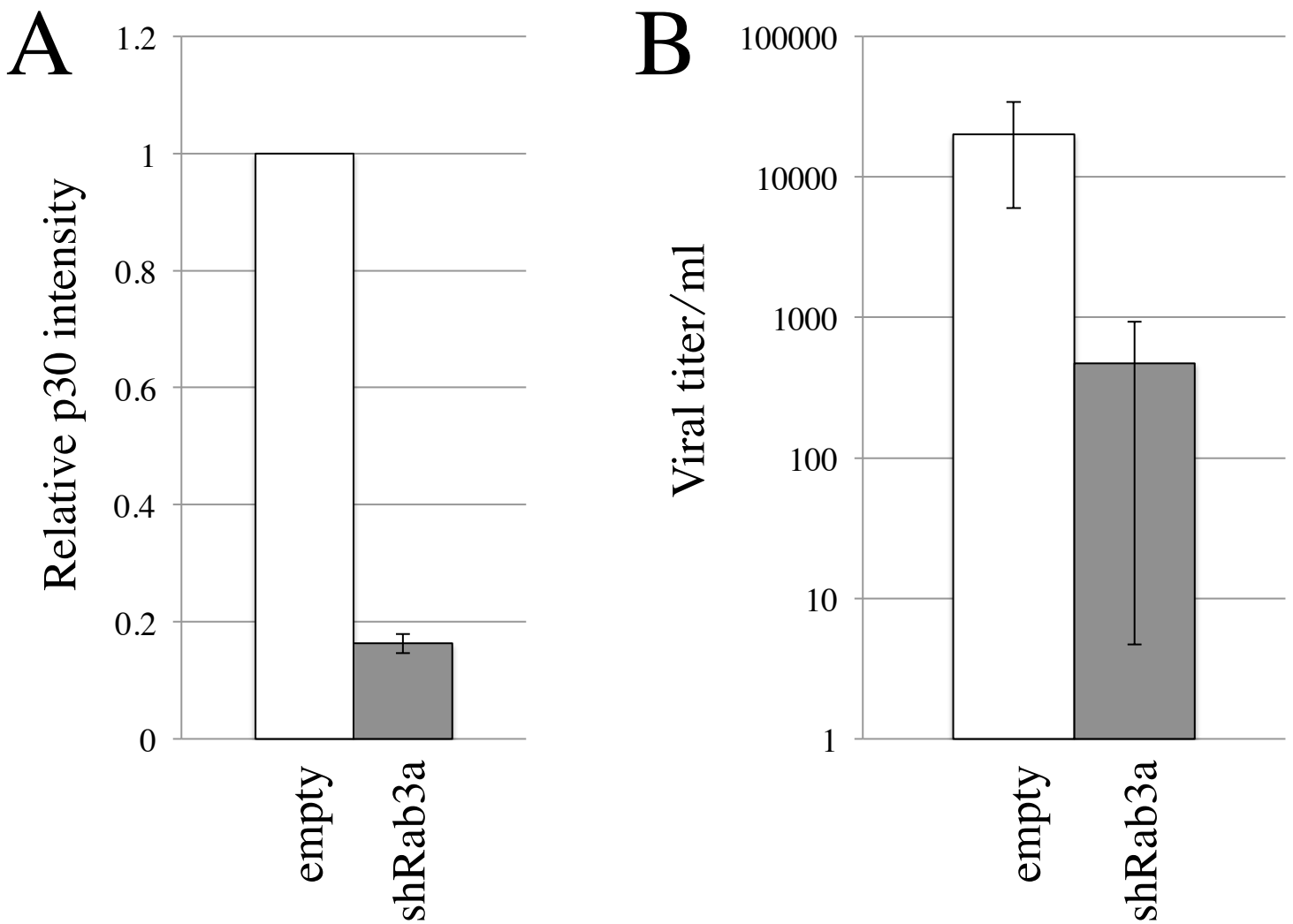

A
Relative p30 intensity
1.2
1
0.8
0.6
0.4
0.2
0
empty
shRab3a
B
Viral titer/ml
100000
10000
1000
100
10
1
empty
shRab3a

Fig. S5

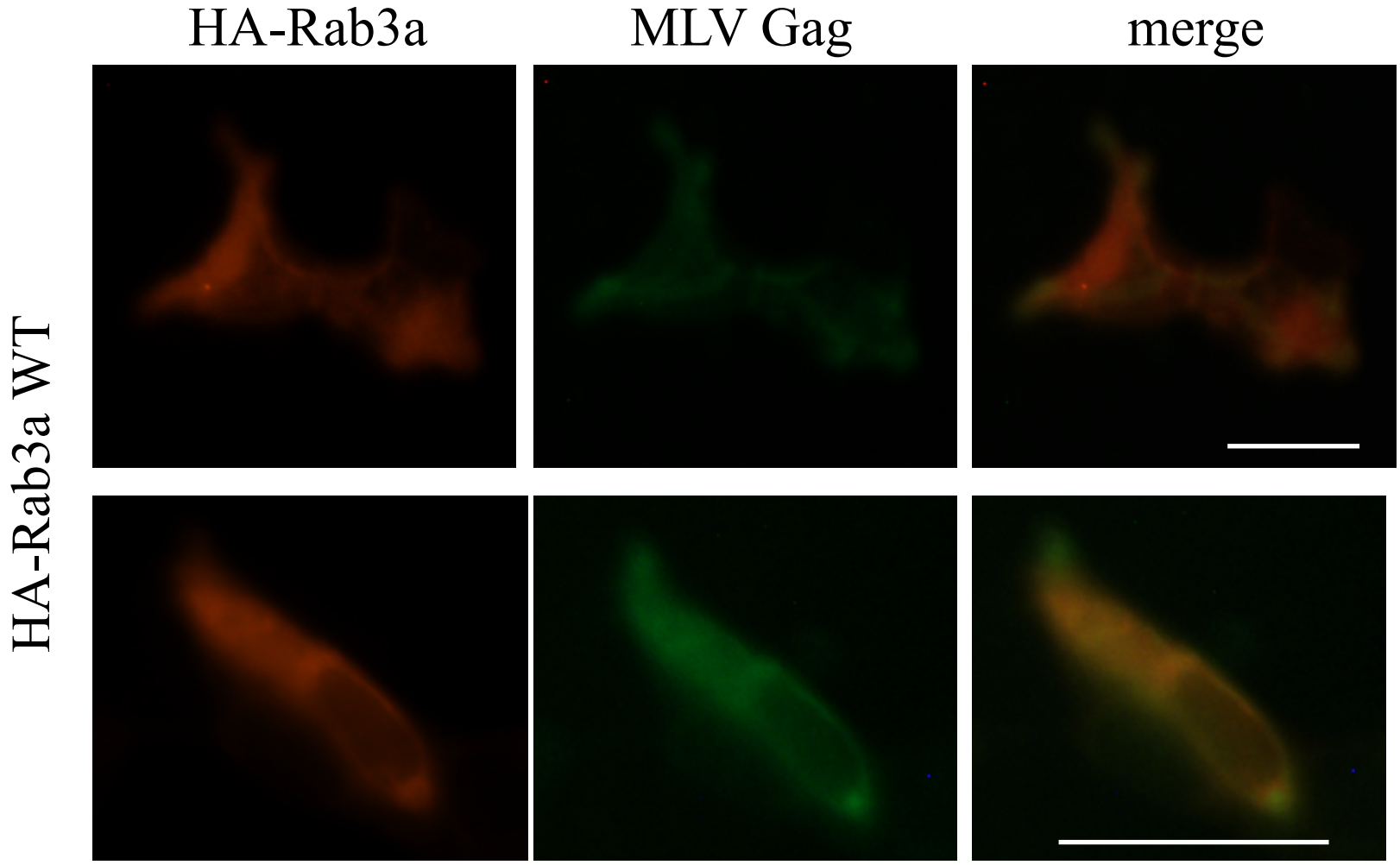


Fig. S6

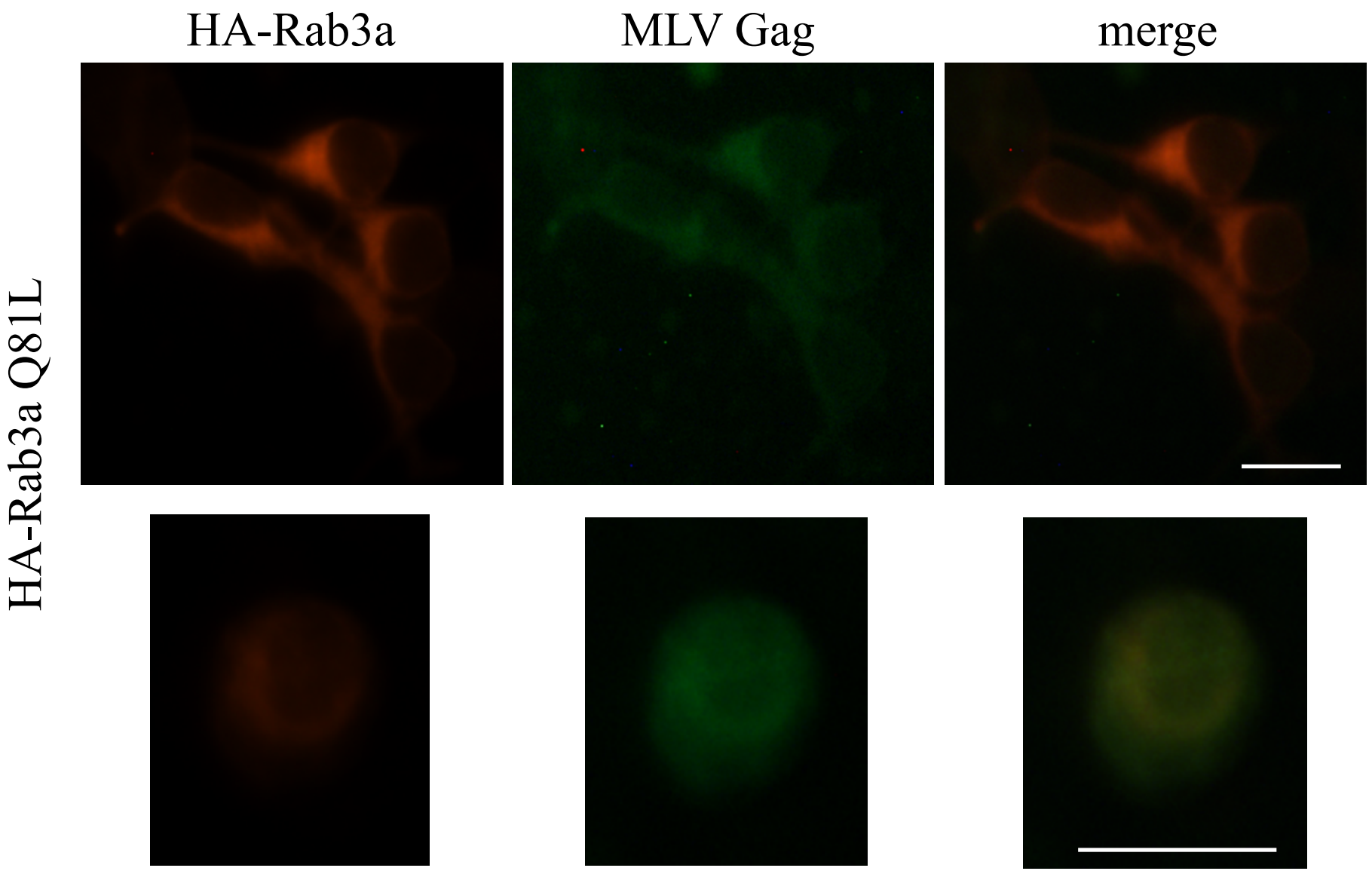
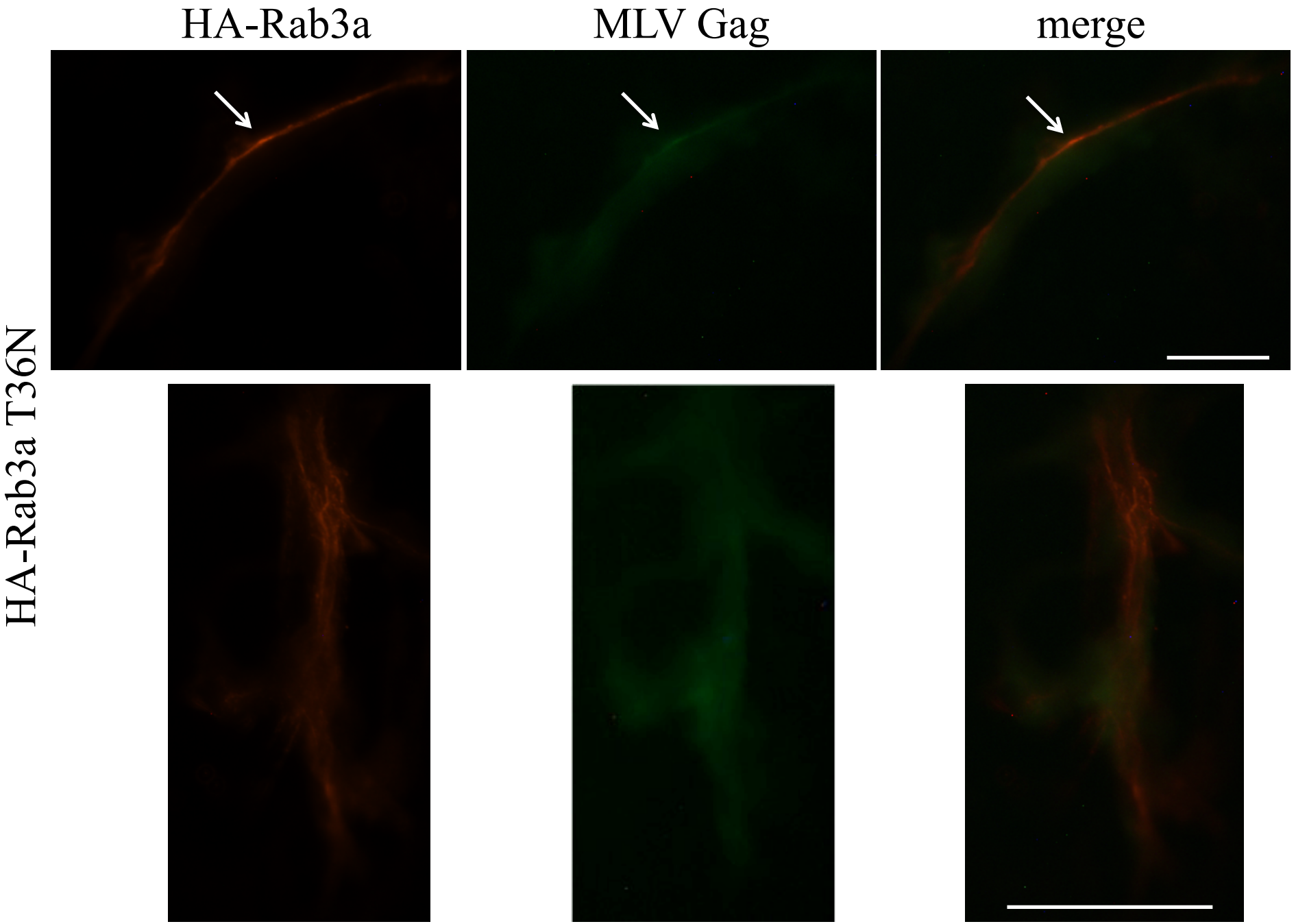


Fig. S7



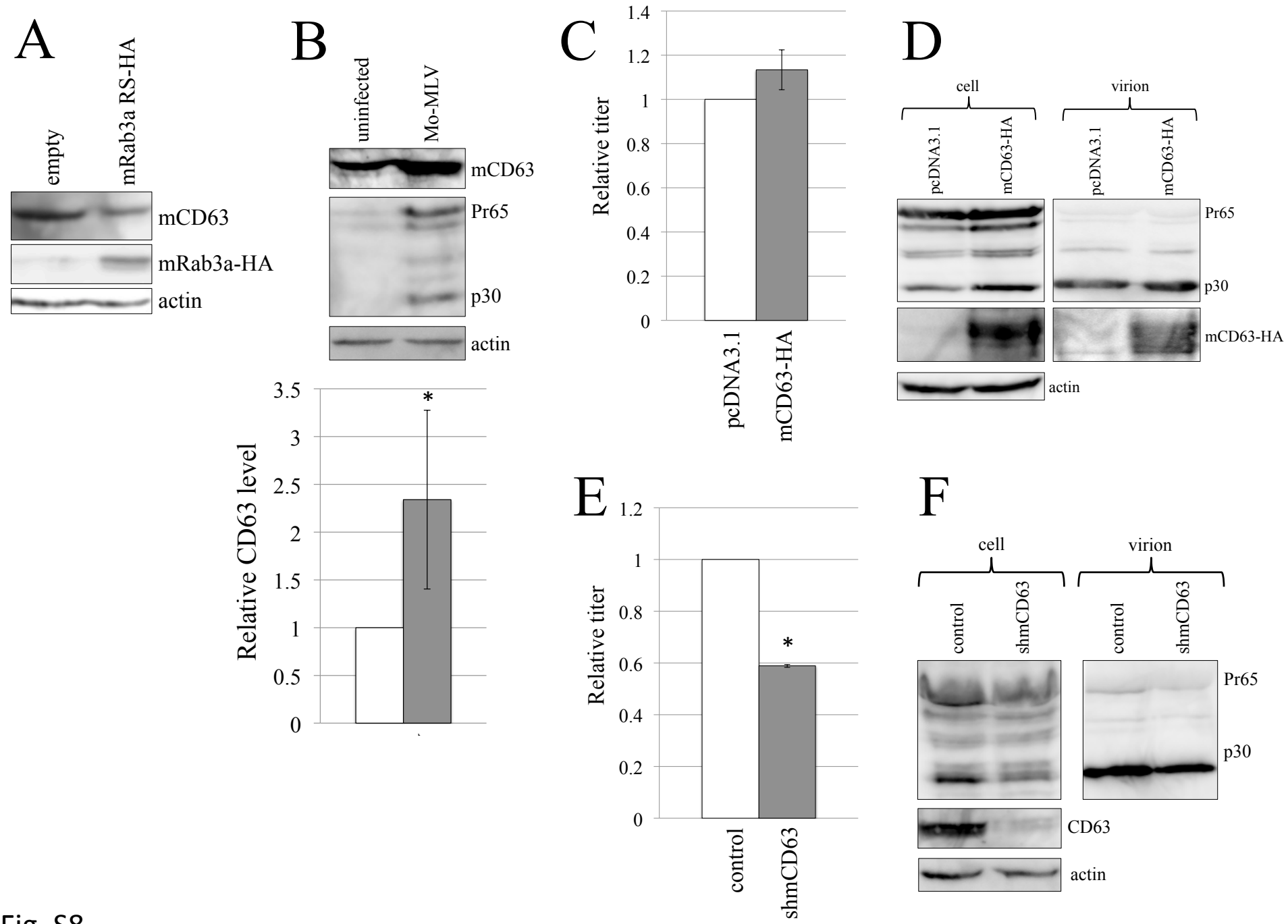

A
empty
mRab3a RS-HA
mCD63
mRab3a-HA
actin
B
uninfected
Mo-MLV
mCD63
Pr65
p30
actin
Relative CD63 level
3.5
3
2.5
2
1.5
1
0.5
0
*
C
Relative titer
1.4
1.2
1
0.8
0.6
0.4
0.2
0
pcDNA3.1
mCD63-HA
D
cell
virion
pcDNA3.1
mCD63-HA
pcDNA3.1
mCD63-HA
Pr65
p30
mCD63-HA
actin
E
Relative titer
1.2
1
0.8
0.6
0.4
0.2
0
*
control
shmCD63
F
cell
virion
control
shmCD63
control
shmCD63
Pr65
p30
CD63
actin

Fig. S8

Fig. S9

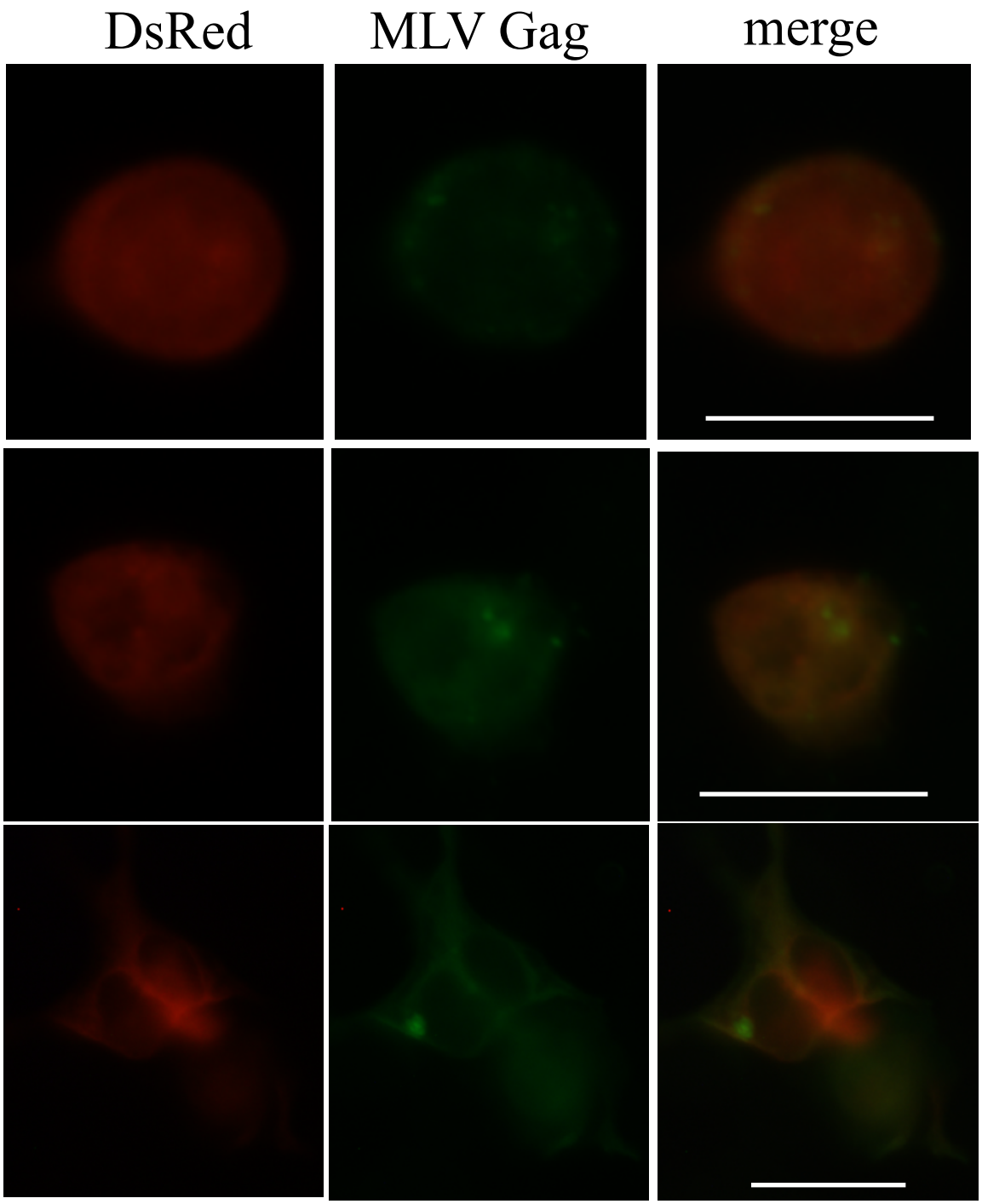
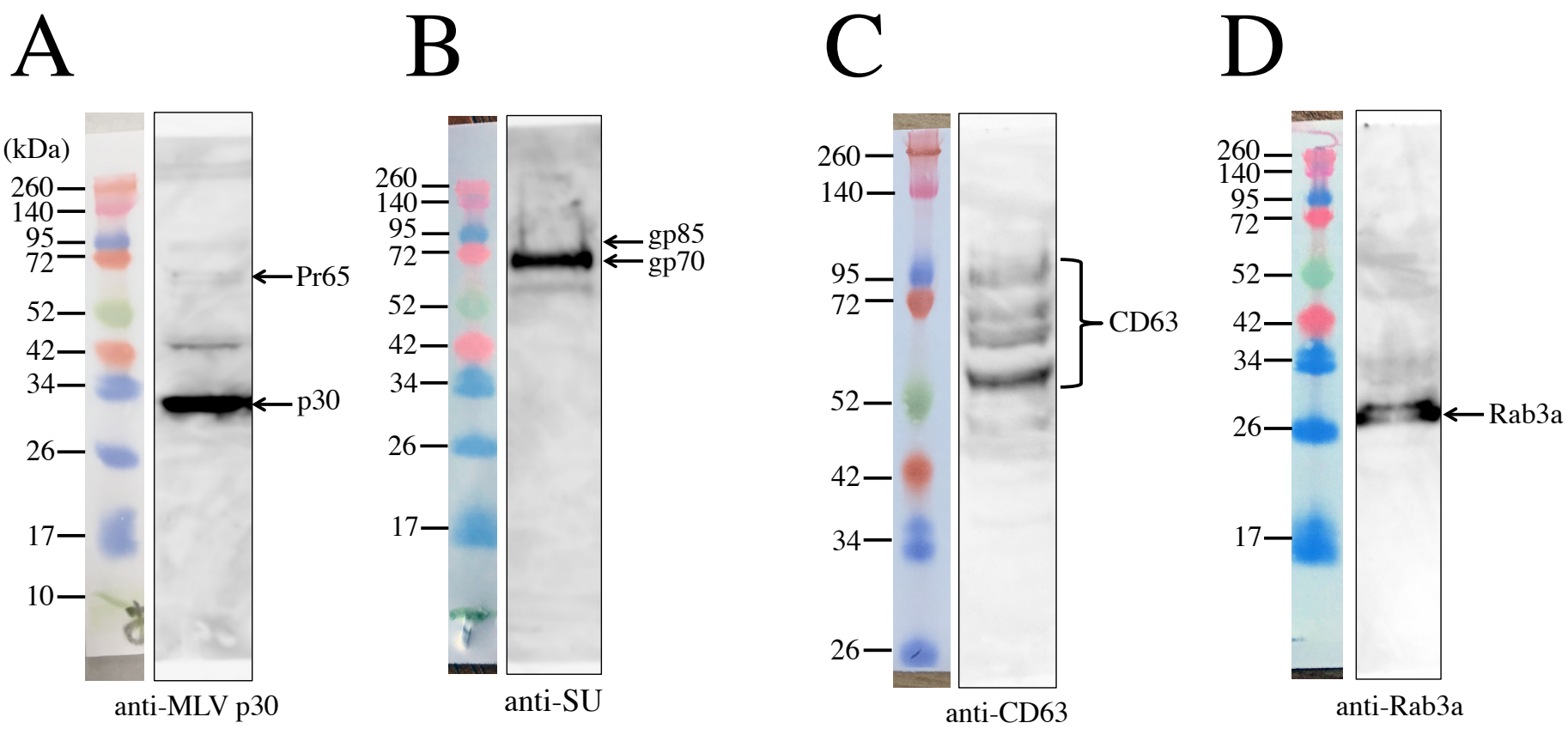


Fig. S10


A
B
C
D
(kDa)
260
140
95
72
52
42
34
26
17
10
Pr65
p30
anti-MLV p30
gp85
gp70
anti-SU
CD63
anti-CD63
Rab3a
anti-Rab3a