The oncolytic avian reovirus p17 protein triggers the IKK/NF-κB signaling by modulating molecular chaperones Hsp90/Cdc37, TRiC/PhLP1 and TRiC/Hsp70 complexes inducing chaperone-mediated autophagy

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ABSTRACT

This study is first to reveal that oncolytic avian reovirus (ARV) modulates the IKK/NF-κB signaling through the Hsp90/Cdc37, T-complex protein-1 ring complex (TRiC)/Hsc70, and TRiC/phosducin-like protein 1 (PhLP1) chaperone complexes, thereby inducing chaperone-mediated autophagy. Specifically, the ARV p17 protein elevates the binding of co-chaperone PhLP1 to TRiC, thereby reducing the complex formation of TRiC/Hsc70 and leading to IκB degradation, suggesting that these tow co-chaperones exhibit a competitive relationship. The p17 protein of ARV plays a crucial role in enhancing the formation of TRiC/PhLP1 complex to protect viral proteins from ubiquitin-proteasome-mediated degradation. Interestingly, we found that ARV p17 enhances the interaction between the Hsp90/Cdc37 chaperone complex and IKK through activation of CK2, which in turn activates NF-κB. Moreover, ARV p17 transcriptionally upregulates Beclin1 and increases the formation of Beclin1/PtdIns3K complex through the CK2/Hsp90/Cdc37 pathway. Immunofluorescence staining reveals that ARV p17 promotes the formation of GFP-LC3 puncta in both Vero and A549 cell lines while a significant reduction in GFP-LC3 puncta was observed in CK2 and Hsp90 knockdown cells. Interestingly, *in situ* proximity ligation assays indicated that ARV p17 promotes the interaction between LC3-II and the cytosolic chaperonin complex (CCT) 2, triggering chaperone-mediated autophagy. This study provides novel insights into ARV-modulated suppression of IκB by regulating co-chaperone PhLP1 and Hsc70 binding to TRiC and activation of the CK2/Hsp90-Cdc37/IKK/NF-κB pathway to induce chaperone-mediated autophagy benefiting viral replication.

KEYWORDS: Avian reovirus, p17, NF-κB, T-complex protein-1 ring complex, Hsp90/Cdc37 complex, TRiC/PhLP1 complex, chaperone-mediated autophagy

Introduction

NF-κB is a family of transcription factors involved in regulating the expression of various genes, including those related to immune responses, inflammation, cell proliferation, and apoptosis [1, 2]. The activation of NF-κB is the result of multiple cellular signaling pathways and plays a crucial role in several viral infections. In the classical pathway, NF-κB activation is typically triggered by pro-inflammatory cytokines including TNF-α and IL-1β [3, 4]. This process involves the phosphorylation and degradation of IκB, allowing the NF-κB heterodimer to be released and translocate into the nucleus. On the other hand, the non-canonical pathway primarily involves the activation of NF-κB-inducing kinase (NIK) and IKKα, which influence the processing of the p100 protein. This leads to the generation of the p52/ RelB heterodimer [5, 6]. A previous study has suggested that short wavelength UV light induces NF-κB activation through casein kinase 2 (CK2)-mediated phosphorylation and IκB degradation [7]. The NF-κB complex, which remains inactive under normal conditions, can be activated by

a range of stimuli such as viral and bacterial infections, proinflammatory cytokines, mitogens, growth factors, and stress-inducing factors [8, 9]. Cellular autophagy is commonly initiated by the deactivation of mTORC1, leading to the activation of the ULK1 kinase complex [10]. Moreover, autophagy regulation involves energy sensors like AMPK and several other signaling pathways, including the PI3K-Akt pathway [11, 12]. Autophagy may generate a cellular environment that facilitates survival in various ways. In addition to its role in normal cellular metabolism and waste removal, autophagy is vital for providing protection in contexts such as pathogen infections and cellular stress [13]. In most cells, three types of autophagy coexist: microautophagy, endosomal microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy [14, 15]. The NF-κB transcription factor family not only regulates cell survival, apoptosis, and inflammation but also contributes to the regulation of autophagy [16-18]. IKK/NF-κB has been found to induce autophagy by directly stimulating the expression of key components of the autophagy machinery, including Beclin 1, ATG5, and LC3 [19, 20]. Conversely, NF-κB can suppress autophagy by upregulating autophagy inhibitors such as Bcl-2 family proteins and the PTEN/mTOR signaling pathway [21, 20]. The activation of NF-κB can stimulate the production of antiviral cytokines, such as IFN-β, which plays a key role in cellular antiviral defense. However, some viruses can exploit NF-κB-induced gene expression to enhance their own replication [8, 22]. For example, the Tat protein of HIV-1 interacts directly with NF-κB, enhancing p65 transcriptional activity and thereby stimulating the viral LTR promoter [23]. The X protein of Hepatitis B virus (HBV) triggers NF-κB activation by stimulating the IKK complex, promoting the expression and replication of viral genes [24]. The NS1 protein of influenza virus blocks NF-κB activation through inhibition of RIG-I and TRIM25, facilitating viral evasion from immune surveillance [25]. ICP0, an

E3 ligase found in herpes simplex virus 1 (HSV-1), contains a RING finger domain at its N-terminus [26]. This viral protein interacts with p50, tagging it for ubiquitination, which results in the suppression of NF-κB activity [27].

Chaperones in eukaryotic cells are classified into the heat shock protein family and the T-complex protein-1 ring complex (TRiC). Chaperone and co-chaperone complexes play a role in the refolding of nascent and misfolded proteins, as well as in promoting the degradation of ubiquitinated proteins through the proteasome [28]. Various forms of stress, such as heat shock, oxidative stress, viral and bacterial infections, and chemical exposure, induce the expression of highly conserved heat shock proteins [29, 30]. The heat shock proteins play a crucial role in regulating multiple signaling cascades, helping to preserve cellular homeostasis. Protein kinases are frequent client proteins of the Hsp90 chaperone, which oversees their formation, stability, and enzymatic activity [31]. Recent studies indicate that Hsp70 and Hsp90 proteins modulate the IKK complex, which is a key activator of the NF-κB signaling pathway [32, 33]. TRiC, also known as chaperonin containing TCP-1 (CCT), is an essential eukaryotic chaperonin with a double-ring structure, each ring consisting of eight subunits (CCT1-8) [34, 35]. The main function of XXX is folding newly synthesized proteins with β-sheet topology, including tubulin, actin, and cellular proteins [36, 37]. Recent findings suggested that TRiC plays a role in the folding of several viral proteins for different stages of the viral life cycle [38-40]. Muscovy duck reovirus p10.8-induced cell cycle arrest and apoptosis depend on the involvement of Cdc20 and the TRiC chaperonins [38]. In mammalian reovirus (MRV) replication, TRiC assists in folding the viral σ3 outer-capsid protein, allowing its assembly into viral particles [39]. Another study highlights the crucial role of the CCT chaperonin in the processing and intracellular transport of HPV particles, as well as in subsequent

126   entry during infection [40].

127   ARV is a double-stranded RNA virus with a genome consisting of ten discrete

128   double-stranded RNA segments, encoding five non-structural proteins and ten

129   structural proteins [41, 42]. Previous studies have demonstrated that the ARV p17

130   protein modulates multiple cellular signaling pathways [43-49]. The ARV p17 protein

131   shuttles between the nucleus and cytoplasm [43, 44], modulating multiple cellular

132   signaling pathways [45-47], and leading to translation shutdown [47], cell cycle

133   retardation [48, 49], and autophagosome formation [45, 46], all of which enhance viral

134   replication. Recently, our team has demonstrated that the p17-modulated molecular

135   chaperone Hsp90/Cdc37 enhances virus replication by protecting viral proteins (i.e. σA,

136   σC, and σNS) from ubiquitin-proteasome degradation [50, 51]. The current study aimed

137   to further implore the mechanisms by which the ARV p17 proteins modulates the

138   IKK/NF-κB signaling through the molecular chaperones TriC and Hsp90 to induce

139   chaperone-mediated autophagy.

140

141   **Results**

142   **The ARV p17 protein enhances the formation of TRiC/PhLP1 complex to reduce**

143   **the binding of co-chaperone Hsc70 to TRiC, leading to IκBα degradation**

144   Previously, we have demonstrated that ARV regulates chaperones Hsp90 and TRiC to

145   stabilize viral proteins, which in turn facilitates viral replication and assembly [50, 51].

146   In this study, we advanced our findings that ARV modulates IκBα/IKK signaling via

147   the chaperones, resulting in promotion of viral replication. Initially, we identified that

148   the TRiC/Hsc70 chaperone complex interacts with IκBα, suggesting that TRiC/Hsc70

149   protects IκBα from ubiquitin-proteasome-mediated degradation. As shown in Figure

150   1A-1B, Co-IP results demonstrate that ARV infection and p17 transfection increase the

6

151 formation of CCT2/PhLP1 complex to stabilize viral proteins and reduce the amount

152 of CCT2/Hsc70 complex. This resulted in reduced the level of IκBα binding to

153 CCT2/Hsc70, leading to decreased levels of IκBα. The tubulin binding to CCT2 was

154 also examined as a positive control [36]. In contrast, inhibition of TRiC activity by

155 HSF-1 (served as a negative control), resulted in a decreased level of IκBα and viral

156 proteins σC and σA expression [50, 51]. Subsequently, we used CCT2 antibody

157 immunoprecipitation to observe its interactions with different co-chaperones.

158 According to the results, after 24 hours of ARV infection or p17 transfection,

159 immunoprecipitation with a CCT2 antibody revealed a significant decrease in binding

160 with IκBα and Hsc70, whereas an increase in binding with PhLP1 was observed (Figure

161 1B). These results demonstrate that ARV p17 modulates the interaction between co-

162 chaperone PhLP1 and TRiC to stabilize viral proteins σC and σA [51]. This finding

163 also demonstrates that CCT2's interactions with viral proteins and IκBα is mediated

164 through a competitive mechanism. Furthermore, when performing

165 immunoprecipitation using an IκBα antibody, a decrease in the interaction between

166 TRiC and IκBα was observed in ARV-infected cells (Figure 1C). These findings

167 demonstrate that ARV enhances the expression levels of its viral proteins by

168 modulating TRiC activity, accompanied by a decrease in the IκBα level. Previous

169 reports suggested that phosphorylation of PhLP is mediated by the protein kinase CK2,

170 and CCT works in concert with PhLP [52-54]. To further study the roles of CK2, PhLP1,

171 and Hsc70 in regulating IκBα and to investigate the interaction between CCT2 and the

172 target proteins, both CK2 and Hsc70 were depleted using target-specific shRNAs,

173 followed by ARV infection. The results interpretation revealed that ARV enhances the

174 association of the σC protein of ARV with CCT2, leading to a reduction in the

175 interaction between CCT2 and IκBα (Figure 1D). In the CK2 shRNA-transfected cells,

the interaction of ARV σC protein with CCT2 was reduced, consequently increasing

the association between CCT2 and IκBα. Conversely, transfection with Hsc70 shRNA

resulted in increased association of σC with CCT2 and reduced interaction with IκBα

(Figure 1D). Furthermore, by utilizing the Hsc70 and CK2 inhibitors, we identify that

Hsc70 only interacted with IκBα and failed to bind to σC protein. This is the first report

suggesting that ARV p17 inhibits IκBα binding to the TRiC/Hsc70 complex and

modulates the TRiC/PhLP1 chaperone complex to stabilize viral proteins. Interestingly,

PhLP1 was only observed in immunoprecipitation with σC antibody and interaction

with IκBα was not identified (Figure 1E). This suggests that the TRiC/PhLP1 complex

protects σC, but not IκBα, from ubiquitin-proteasome degradation,. The findings

demonstrate that CCT2 associates with σC and IκBα through different co-chaperones.

To further study whether TRiC, Hsp70, and CK2 affect virus replication, inhibition of

TRiC by HSF-1 and CK2 by TBB was performed. Our results reveal that suppression

of TRiC and CK2 significantly reduced virus yields (Figure 1F) and no changes were

observed in VER-155008-treated cells (Hsc70 inhibitor).

ARV p17 enhances the phosphorylated form of CK2

Having shown that ARV infection and p17 transfection modulate the binding of PhLP1

to CCT2, thereby stabilizing viral proteins, we next aimed to investigate whether ARV

infection and p17 transfection regulate the expression levels of co-chaperones of TRiC.

Previously, it has been reported that CK2 regulates TRiC activity and promotes its

interaction with PFDN5 to assist in protein folding [55]. Following 24-hour ARV post

infection or p17 transfection, whole cell lysates were collected for Western blot analysis

to observe protein expression levels. The results indicate that ARV infection and p17

transfection did not alter the expression levels of co-chaperones Hsc70, PhLP1, and

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201 PFDN5, however, resulted in an increase in the phosphorylation levels of p-CK2 in a

202 time-dependent manner (Fig. 2).

203

204 **ARV induces chaperone-mediated autophagy in Vero cells**

205 In most cells, three types of autophagy coexist including microautophagy, endosomal

206 microautophagy, chaperone-mediated autophagy, and macroautophagy [15].

207 Interestingly, *in situ* proximity ligation assays indicate that ARV infection and p17

208 transfection promoted the interaction between LC3-II and CCT2, triggering chaperone-

209 mediated autophagy (Figure 3A). Additionally, the interaction of LC3-II with CCT2

210 was significantly reduced in cells treated with CK2 and Hsc70 inhibitors (Figure 3A-

211 3B). According to existing reports, the formation of a complex involving several

212 proteins is necessary to induce chaperone-mediated autophagy [56]. Following 24

213 hours of ARV infection, immunoprecipitation assay was performed using CCT2

214 antibodies. The results showed that ARV promotes the interaction of Lamp2 and LC3-

215 II with CCT2 (Figure 3C), but reduces the amount of IκBα binding to CCT2.

216 Additionally, immunofluorescence staining confirmed the findings, demonstrating that

217 ARV enhances the interaction between LC3-II and CCT2, which triggers chaperone-

218 mediated autophagy (Figure 3D).

219

220 **ARV p17 acts as a NF-κB activator**

221 To investigate whether ARV infection and p17 transfection activate NF-κB at

222 different time points in Vero cells, whole cell lysates were collected for cytoplasmic

223 and nuclear protein isolation followed by Western blot analysis to evaluate the levels

224 of p-IκBα and localization of p65 and p50 within the nucleus. The results demonstrate

225 that the phosphorylation of IκBα (Ser32) is increased by both ARV infection and p17

226   transfection in a time-dependent manner. This in turn reduced the cytoplasmic levels

227   of p65 and p50 and increased the levels of p65 and p50 in the nucleus, suggesting that

228   ARV p17 suppresses IκBα to promote p65 and p50 translocation into the nucleus

229   (Figure 4), thereby activating NF-κB. Interestingly, the increased levels of p-IκBα

230   peaked at two different time points (at 6 and 18 hours post infection). The finding

231   corresponds to our previous report confirming the increased level of LC3-II in ARV-

232   infected cells at 9- and 24-hours post-infection [45].

233   **The ARV p17 protein modulates protection of IKK from ubiquitin-proteasome-**

234   **mediated degradation through the CK2/Hsp90-Cdc37 signaling and enhances IκB**

235   **degradation**

236   Having shown that the phosphorylation of IκBα (Ser32) increased in the ARV-infected

237   or p17-transfected cells, we next wanted to confirm whether p17 modulate the

238   CK2/Hsp90-Cdc37 signaling to protect IKK from ubiquitin-proteasome-mediated

239   degradation. Vero cells were infected with ARV at a MOI of 10 or transfected with p17

240   followed by co-transfection with CK2 shRNA. After 24 hours, whole cell lysates were

241   collected for immunoprecipitation using an Hsp90 antibody to analyze protein

242   interactions. As shown in Figure 5A, both ARV infection and p17 transfection

243   significantly enhanced the interaction among Hsp90, Cdc37, and IKK. The amount of

244   IKK binding to the Hsp90/Cdc37 complex was significantly reduced in CK2

245   knockdown cells. In order to confirm that the interaction between IKK, Hsp90, and

246   Cdc37 in preventing protein degradation, the ubiquitination of IKK and IκBα was

247   analyzed. Vero cells were transfected with p17 expression plasmid and treated with 17-

248   AAG, an Hsp90 inhibitor, for 24 hours. Cell lysates were collected and

249   immunoprecipitation was performed using IKK and IκBα antibodies, respectively,

250   followed by Western blot analysis for IKK and IκBα ubiquitination. The results

revealed that p17 transfection led to a reduction in IKK ubiquitination, accompanied by

an increase in IκBα ubiquitination (Figure 5B). It was confirmed that ARV p17

facilitates the translocation of NF-κB into the nucleus (Figure 4). Immunoprecipitation

results indicate that ARV p17 was unable to significantly reduce IKK ubiquitination in

Hsp90 inhibitor (17-AAG)-treated cells and a reduction in IκBα ubiquitination was

observed (Figure 5B). These findings suggest that the protein of ARV modulates

protection of IKK from ubiquitin-proteasome-mediated degradation through the

CK2/Hsp90-Cdc37 signaling.

The ARV p17 protein induces autophagy through the Hsp90-Cdc37/IKK/NF-kB pathway

Having demonstrated that ARVp17 protein protects IKK from ubiquitin-proteasome-

mediated degradation through the CK2/Hsp90-Cdc37 signaling, we next wanted to

investigate the role of CK2 in nuclear translocation of NF-κB. ARV-infected cells were

treated with the CK2 inhibitor (i.e. TBB). After 24 hours of incubation, whole cell

lysates were harvested for analyzing nuclear translocation of p65 and p50 proteins. The

data indicate that ARV enhances the nuclear translocation of p65 and p50 subunits of

NF-κB (Figure 6A). However, in the presence of TBB, which inhibits CK2, the nuclear

translocation of p65 and p50 was significantly reduced (Figure 6A), demonstrating that

ARV mediates NF-κB translocation via the CK2/Hsp90-Cdc37 pathway. Furthermore,

to investigate whether Hsp90 modulates NF-κB translocation, cells were infected with

ARV or transfected with pCI-Flag-p17 followed by treatment with the Hsp90 inhibitor

17-AAG and the NF-κB inhibitor Bay11-7085. After 24 hours, cytoplasmic and nuclear

proteins were collected to analyze the translocation of p65 and p50 proteins. The results

showed that in the ARV-infected or p17-transfected cells, there was an increase in

276   cytoplasmic levels of IKK and a reduction in the levels of IκBα (Figure 6B). When

277   Vero cells were treated with the Hsp90 inhibitor 17-AAG, the ARV- and p17-mediated

278   translocation was significantly diminished in 17-AAG and Bay11-7085-treated cells

279   (Figure 6B). The findings demonstrate that ARV infection and p17 protein modulate

280   the IKK and IκBα levels through the CK2/Hsp90-Cdc37 pathway, thereby regulating

281   the translocation of p65 and p50 subunits of NF-κB. It has previously been shown that

282   the translocation of NF-κB into the nucleus regulates the expression of various genes,

283   promoting survival, proliferation, inflammation, immune regulation, and autophagy [4].

284   Therefore, we further investigated whether ARV regulates the expression of the key

285   autophagy protein (i.e. Beclin1) through the NF-κB. Following ARV infection, cells

286   were treated with different concentrations of the Bay11-7085 inhibitor. After 24 hours,

287   cell lysates were collected for analysis through RT-PCR and Western blot. As shown

288   in Figure 6C, the results demonstrated that ARV dramatically enhances the mRNA and

289   protein levels of Beclin1, suggesting that ARV transcriptionally upregulates Beclin1.

290   However, upon the addition of Bay11-7085, the mRNA and protein levels of Beclin1

291   were significantly reduced, confirming that ARV elevates expression level of Beclin1

292   *via* the NF-κB pathway.

293

294   **ARV p17 enhances the PtdIns3K/Beclin1 complex formation leading to induction**

295   **of autophagosome formation**

296   After demonstrating that the ARV p17 protein modulates NF-κB translocation through

297   the CK2/Hsp90-Cdc37 pathway, we next explored whether the CK2/Hsp90-Cdc37

298   pathway plays a role in the formation of PtdIns3K/Beclin1 complex. Vero cells were

299   transfected with the pCI-Flag-p17 plasmid for six hours followed by transfection of

300   CK2 shRNA or the pCI-neo-CK2 plasmid. After 24 hours of co-transfection, cell

301 lysates were collected for Western blot analysis. In the p17-transfected cells, the levels

302 of p-Cdc37 (Ser13), Beclin1, and LC3-II showed significant increases (Figure 7A),

303 while these enhancements were notably suppressed in the CK2 shRNA co-transfected

304 cells (Figure 7A). Interestingly, when pCI-Flag-p17 and pCI-neo-CK2 plasmids were

305 co-transfected, the expression levels of p-Cdc37 (Ser13), Beclin1, and LC3-II were

306 significantly increased (Figure 7A), suggesting that ARV p17 induces autophagy via

307 the CK2/Hsp90-cdc37 pathway. However, when the Hsp90 inhibitor 17-AAG or the

308 NF-κB inhibitor Bay11-7085 was used, ARV p17 failed to increase the Beclin1 and

309 LC3-II expression levels (Figure 7B), further confirming that the ARV p17 protein

310 enhances autophagy through the CK2/Hsp90-Cdc37 pathway. Cellular autophagy

311 appears through the assembly of multiple proteins into a complex that drives the

312 autophagic formation [15]. Thus, we utilized immunoprecipitation to investigate the

313 status of the autophagy protein complex in cells after ARV infection. As shown in

314 Figure 7C, while using class III PI3K or Beclin1 as immunoprecipitation antibodies,

315 the formation of the PtdIns3K and Beclin1 complex was significantly increased in the

316 p17-transfected cells. However, upon co-transfection of CK2 or Hsp90 shRNA, the

317 interaction between PtdIns3K and Beclin1 was decreased, further supports the that p17

318 induces autophagy formation through the CK2/Hsp90 pathway.

319

320 **The ARV p17 protein induces autophagosome formation through the CK2/ Hsp90**

321 **pathway**

322 To analyze autophagosome formation, cells were transfected with a LC3-GFP fusion

323 protein, acting as a fluorescent indicator for autophagosomes. LC3-I shows a diffuse

324 staining pattern in the cytoplasm, while LC3-II appears as distinct punctate staining

325 [57]. In both ARV-infected and p17-transfected Vero cells, a substantial increase in

13

326   LC3-GFP puncta was observed (Figure 8A). However, co-transfection with Hsp90 or

327   CK2 shRNA led to a marked reduction in LC3-GFP puncta (Figure 8A). A comparable

328   trend was noted in thapsigargin (TG)-treated Vero cells compared to the mock control.

329   Correspondingly, the quantitative data of the average LC3-GFP puncta per cell showed

330   the same trends (Figure 8B).

331

332   ARV p17-induced autophagy through the Hsp90/NF-kB pathway in A549 cancer

333   cells

334   We have confirmed that ARV p17 induces autophagy by modulating Beclin 1/PtdIns3K

335   complexes through the CK2/Hsp90/NF-κB pathway in Vero cells. Previous studies

336   have identified ARV as an oncolytic virus [58], leading us to further investigate whether

337   ARV p17 exhibits similar functions in A549 cancer cell lines. A549 cells were infected

338   with ARV for six hours, followed by transfecting with CK2 and Hsc70 shRNA. After

339   24 hours, cell lysates were collected, and CCT2 immunoprecipitation was performed to

340   analyze protein interactions. The data revealed that ARV facilitates the binding of viral

341   protein σC to CCT2, resulting in a decreased interaction between CCT2 and IκBα in

342   A549 cancer cells (Figure 9A). Upon transfection with CK2 shRNA, the ARV σC-

343   CCT2 interaction was weakened, resulting in a higher association between CCT2 and

344   IκBα. In contrast, silencing of Hsc70 using Hsc70 shRNA enhanced the binding of σC

345   with CCT2 while reducing its interaction with IκBα (Figure 9A). After ARV infection

346   of A549 cells for 24 hours, cell lysates were collected and subjected to

347   immunoprecipitation with an Hsc70 antibody, revealing that Hsc70 interacted

348   exclusively with IκBα and showed no binding to σC (Figure 9B). Moreover, A549 cells

349   were infected with ARV or transfected with pCI-Flag-p17, then co-transfected with

350   CK2 shRNA. Twenty-four hours later, cell lysates were harvested, and

351 immunoprecipitation was carried out with an Hsp90 antibody to analyze the

352 interactions between proteins. The findings demonstrate that both ARV infection and

353 p17 transfection substantially promoted the interaction between Hsp90, Cdc37, and

354 IKK, but this interaction was notably reduced upon CK2 shRNA co-transfection

355 (Figure 9C). Next, we applied whether ARV induces autophagy in A549 cancer cells.

356 Immunoprecipitation was used to analyze the state of the autophagy protein complex

357 in A549 cancer cells following ARV infection. Using PtdIns3K as the

358 immunoprecipitation antibody, the pull down revealed that p17-transfected cells

359 substantially increased the formation of the PtdIns3K and Beclin1 complex (Figure 9D).

360 However, co-transfection with CK2 or Hsp90 shRNA reduced this interaction (Figure

361 9D), suggesting that ARV p17 promotes autophagy of A549 cancer cells through the

362 CK2/Hsp90 pathway. The immunofluorescence results in Figure 9E demonstrate that

363 LC3-GFP puncta increased substantially in both ARV-infected and p17-transfected

364 A549 cells. However, co-transfection with Hsp90 or CK2 shRNA notably reduced the

365 LC3-GFP puncta (Figure 9E).

366

367 **Discussion**

368 Viruses depend on host cells for survival, utilizing cellular mechanisms for gene

369 replication and viral particle assembly. Despite notable advancements in research on

370 viral regulation of host cell mechanisms, numerous issues are still unanswered.

371 Previously, we have demonstrated that ARV facilitates viral replication by modulating

372 cellular translation, cell cycle, and autophagy [45, 46, 48, 49]. Recent studies indicated

373 that ARV utilizes TRiC and Hsp90 chaperone systems to support viral proteins

374 synthesis and particle assembly [50, 51]. This study, for the first time, demonstrates

375 that ARV-modulated suppression of IκBα by regulating co-chaperone PhLP1 and

15

Hsc70 and activation of the IKK/NF-κB signaling through TRiC and Hsp90 chaperones to induce chaperone-mediated autophagy benefiting viral replication. The function of CCT is modulated by prefoldin, PhLPs, and Hsc70 to ensure precise control over the protein folding process [36]. However, the folding of multidomain proteins often requires the coordinated action of multiple chaperone classes, which collaborate to provide a safeguarded folding pathway. This indicates that CCT is tightly regulated by co-chaperones for its role in folding actin and other proteins, and the interaction of various cochaperones with CCT determines the outcome of the final folding process. A previous study has confirmed that ARV p17 regulates PTEN activity via CK2 in the cytoplasm [47]. The CK2 is involved in various cellular functions, including cell division, growth, and gene expression [59, 60]. Additionally, CK2 promotes Hsp90 activity through the phosphorylation of Cdc37 [61, 62]. The finding reveals that ARV p17 stimulates phosphorylation of Cdc37 at Ser13, which in turn facilitates its binding with Hsp90 and reinforces Hsp90 activity. However, when we used the inhibitor TBB to suppress CK2, ARV p17 was unable to effectively phosphorylate Cdc37 at Ser13, demonstrating that ARV p17 activates the Hsp90/Cdc37 complex via CK2 [50]. Conversely, the P protein of rabies virus requires assistance from Hsp90 but is not dependent on the formation of the Cdc37/Hsp90 complex. Even without Cdc37 phosphorylation at Ser13, Hsp90 is still able to support the expression and stability of the P protein of the rabies virus [63].

TRiC plays a crucial role in MRV replication by assisting the folding of the viral σ3 outer-capsid protein, which is essential for its assembly into viral particles [39]. Although we demonstrated that p17 protein of ARV modulates molecular chaperone TRiC to protect outer-capsid protein σC and inner core protein σA and non-structural protein σNS from ubiquitin- proteasome degradation [51], it is still unclear which co-

chaperone interacts with TRiC to stabilize viral proteins. This study further demonstrated that p17 protein of ARV enhances the interaction between PhLP1 and CCT2 via CK2-mediated phosphorylation of PhLP1[52], suggesting that p17 plays an important role in modulating the formation of TRiC/PhLP1 complex [53, 54], which chaperones the σC and σA proteins of ARV [51]. This is the first report to reveal a regulatory role for PhLP1 in CCT2-dependent folding or stabilization of viral proteins. The current and past studies reveal that Hsp90/Cdc37 and TRiC/PhLP1 chaperone complexes are essential for chaperoning p17, σC, σA, and σNS proteins of ARV, respectively. The binding of PhLP1 to CCT2 diminishes Hsc70's association with TRiC, leading to IκBα degradation, thereby subsequent activation of NF-κB. This study suggests that PhLP1 and Hsc70 exhibit a competitive relationship in binding to TRiC, with ARV stimulation causing a shift in their balance. The current study provides the first evidence to reveal that the TRiC/Hsc70 chaperone machinery plays a critical role in protecting IκBα from ubiquitin-proteasome-mediated degradation.

Previous studies have confirmed that Hsp90 enhances the activation of the transcription factor NF-κB [64, 65]. In the early stages of ARV infection, NF-κB activation is enhanced to delay the onset of apoptosis [66]. The present study demonstrates that p17 protein of ARV indeed activates the IKK/NF-κB signaling through the CK2/Hsp90/Cdc37 pathway, which in turn induces chaperone-mediated autophagy. Hsp90 activates NF-κB by enhancing the stability of the IKK protein, which leads to phosphorylation and ubiquitination of IκBα, ultimately resulting in its degradation [67]. Previous studies have suggested that several viruses, such as human immunodeficiency virus (HIV), human T-lymphotropic virus 1 (HTLV-1), hepatitis B virus, and influenza virus, can trigger NF-κB activation. The activated NF-κB supports viral replication, postpones virus-induced cell death, or aids in immune response

426   mediation [8, 68]. In some dsRNA viruses, NF-κB is activated via the accumulation of

427   viral RNA, which subsequently activates the dsRNA-dependent kinase PKR [69, 70].

428   An earlier study has demonstrated that the X protein of HBV regulates cellular signaling

429   pathways by activating NF-κB through Ras and MEKK-1 [71]. HTLV-1's Tax protein

430   has been shown to bind directly with IKK [72, 73], which promotes NF-κB activation,

431   leading to enhanced cellular gene expression that promotes cell growth and transforms

432   cells into malignant tumor cells [8, 74]. This study demonstrates that ARV p17

433   transcriptionally upregulates Beclin1 via the NF-κB signaling, enhancing cellular

434   autophagy. Autophagy, which serves as a cellular defense mechanism, is manipulated

435   by viruses to enhance their replication. When Sindbis virus infects the mouse brain,

436   artificially increasing Beclin1 expression significantly reduces viral titers [75]. The

437   VP1 protein of foot-and-mouth disease virus binds to SQSTM1/p62 and promotes

438   autophagosome formation, facilitating viral entry and replication within host cells [76].

439   Hepatitis C virus stimulates the formation of autophagosomes but blocks their

440   maturation, leading to autophagosomes accumulation and creating a favourable

441   environment for viral replication in the cell [77]. Many viruses have been reported to

442   regulate autophagy to facilitate their replication. The current and past studies from our

443   laboratory have demonstrated that ARV p17 induces autophagy to enhance viral

444   replication [45, 47]. The mechanisms underlying 17-induced chaperone-mediated

445   autophagy benefiting viral replication remain unclear and warrant future investigations

446   to elucidate its mechanism.

447   A model is proposed to illustrate that ARV p17-modulated suppression of IκB by

448   regulating co-chaperone PhLP1 and Hsc70 binding to TRiC and activation of the

449   CK2/Hsp90-Cdc37/IKK/NF-κB pathway to induce chaperone-mediated autophagy

450   (Figure 10). This study sheds additional light on how ARV modulates the cellular

451 autophagy pathway, which may also provide a novel perspective for developing

452 antiviral approaches.

453

454 **Materials and Methods**

455 **Cells and virus**

456 The S1133 strain of ARV was used in this study. Vero and A549 cells were grown in

457 minimum essential medium (MEM) or HAM's F-12K (Kaighn's) medium

458 supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES (pH 7.2) and 1%

459 Penicillin/Streptomycin at 37°C in a 5% CO2 humidified incubator. Once the cells

460 reached a monolayer, they were prepared for passaging under sterile conditions in a

461 laminar flow hood. The cells were washed twice with 1X PBS and then digested using

462 trypsin. After cells were resuspended, fresh culture medium containing FBS was added

463 to neutralize the trypsin activity. The cells were evenly transferred into a 4-well plate

464 for subsequent experiments.

465

466 **Reagents and antibodies.**

467 HSF1A (TRiC inhibitor), TBB (CK2 inhibitor), VER-155008 (Hsc70 inhibitor), and

468 Bay11-7085 (NF-κB inhibitor) were purchased from MedChemExpress (Monmouth

469 Junction, USA). The p17 polyclonal antibodies were produced by our laboratory.

470 Monoclonal antibodies against σA, σC, and σNS proteins of ARV were prepared in

471 our laboratory, as described previously [78, 79]. The primary and secondary antibodies

472 used in this study, along with their catalogue numbers and working dilution are detailed

473 in Table 1.

474

475 **shRNAs used in this study**

476 The pLKO-AS1-puro plasmid encoding shRNAs were obtained from the National

477 RNAi Core Facility, Academia Sinica, Taiwan. The target sequences for casein kinase

478 II and Hsp90 were 5-CGTAAACAACACAGACTTCAA-3 and 5-CGTAAACAACA

479 CAGACTTCAA-3, respectively. The target sequence for Hsc70 was 5-GCTCGATTT

480 GAGGAGTTGAAT-3. The shRNA with the most significant down-regulated effect for

481 a respective gene was selected and used in this study. The cells transfected with the

482 respective shRNA for 6 h, followed by infection with ARV at an MOI of 10 for 24

483 hours, respectively. Whole cell lysates were collected for Western blot analysis.

484

485 **Reverse transcription (RT) and polymerase chain reaction (PCR), plasmid**

486 **construction and transient transfection**

487 The pCI-Flag-p17 constructs were generated as demonstrated previously (13). For the

488 construction of pCI-Flag-CK2 plasmid, gene fragments were amplified by PCR using

489 specific primers shown in Table 2. The total RNA was extracted from ARV-infected

490 Vero cells using TRIzol solution (Thermo Fisher Scientific Inc.) according to the

491 manufacturer's protocol. A total of 3 ul of total RNA was used as templates for RT with

492 M-MLV reverse transcriptase using the respective primers (Table 2) (Promega Co.,

493 Madison, USA). Reverse transcription was carried out at 42°C for 15 min and 72°C for

494 15 min. PCR was performed with 1 μl of cDNA, 1 μl of each primer, 2 μl of PCR mix,

495 and 15 μl of ddH2O, in a total volume of 20 μl. The PCR conditions for amplification

496 were 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 and extension at 72°C for

497 30 s (p17) or 2 min (CK2), followed by 72°C for 10 min for a final extension. The PCR

498 products were electrophoresed in a 1% agarose gel and were then purified using an

499 agarose gel DNA extraction kit (Bio kit, Taiwan). Purified PCR products were digested

500 with the respective enzymes, followed by ligation into the same restriction sites of the

501   pCI-neo-Flag plasmid to generate pCI-Flag- p17 and CK2 plasmids, respectively. All

502   constructs were confirmed by DNA sequencing.

503   For transfection, cells were grown to 70-80%, washed twice with serum-free

504   medium, and supplemented with fresh FBS-free medium. Plasmid DNA was mixed

505   with the transfection reagent (GenJet™, SignaGen Laboratories, USA) following the

506   manufacturer's recommended protocol. The prepared mixture was then added to the

507   culture dish. After 6 hours of transfection, the supernatant with the transfection reagent

508   was removed. The cells were washed twice with serum-free medium, followed by the

509   addition of fresh FBS-containing medium. Cells were maintained in a 37°C incubator

510   with 5% CO_2, and samples were collected at indicated time points for further analysis.

511

512   **Proximity ligation assays**

513   The Duolink commercial assay (Sigma-Aldrich, cat no. DUO 92008) was used to detect

514   the LC3/CCT2 interaction in ARV-infected Vero cells. PLA enables the detection of

515   direct protein-protein interactions within a range of approximately 40 nm in fixed, intact

516   cells [80]. Vero cells were cultured on 18x18 mm coverslips and, after 24 hours of ARV

517   infection, fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells

518   were treated with a blocking buffer for 30 minutes at 37°C, followed by incubation with

519   specific primary antibodies. Following several washing steps, PLA probes linked to

520   anti-mouse and anti-rabbit secondary antibodies were applied to the cells. Next, the

521   cells underwent ligation and amplification reactions. Following two washes with 1 x

522   PBS, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes.

523   The cells were mounted on glass slides using ibidi mounting medium (Ibidi GmbH,

524   Lochhamer Schlag, Germany) and examined using a BX51 fluorescence microscope.

525

Cytoplasmic and nuclear protein isolation

The transfected cells were harvested using trypsin-EDTA and transferred to microcentrifuge tubes. The samples were centrifuged at 800 ×g for 10 minutes at 4°C. After discarding the supernatant, the cells were resuspended in 1X PBS and washed twice using the same step. Cytoplasmic and nuclear protein extraction was carried out using the Compartmental Protein Extraction Kit (CNMCS, BioChain). The washed cells were resuspended in 200 µl of buffer C and agitated at 4°C for 20 minutes. Membrane disruption was achieved by repeatedly drawing the samples (~100 times) through a 26.5-gauge syringe. The lysates were then centrifuged at 15,000 ×g for 20 minutes at 4°C, and the supernatant was collected as the cytoplasmic protein fraction. The pellet was resuspended in 400 µl of buffer W, mixed thoroughly, and shaken at 4°C for 5 minutes. The mixture was then centrifuged at 15,000 ×g for 20 minutes at 4°C, and the supernatant was discarded. The pellet was resuspended in 100 µl of buffer N and shaken at 4°C for 20 minutes. The mixture was then centrifuged at 15,000 ×g for 20 minutes at 4°C, and the resulting supernatant was collected as the nuclear protein fraction.

Co-immunoprecipitation assays

The Catch and Release® v2.0 kit was used in this study (Millipore Corporation, Billerica, Massachusetts, USA). The cells were washed twice with 1x PBS and lysed in 200 µl of CHAPS lysis buffer (40 mM HEPES [pH 7.5], 1 mM EDTA, 10 mM glycerophosphate, 120 mM NaCl, 50 mM NaF, 10 mM pyrophosphate, and 0.3% CHAPS). 1000 µg of total proteins collected from each sample were incubated with 2 µg of the indicated antibodies or rabbit IgG (negative control). Sample proteins, antibodies, and resin were sequentially incubated together and incubated at 4°C for 12h

following the manufacturer's instructions. After removing the supernatant, add 400 µl

of 1X Wash buffer to the column. Centrifuge at 4°C, 2000 xg for 30 seconds, discard

the filtrate, and repeat the washing step three times. Finally, add 70 µl of Elution Buffer,

mix gently, and incubate on ice for 5 minutes. Centrifuge at 4°C, 2000 xg for 30 seconds,

and collect the supernatant as the sample for analysis. The immunoprecipitated proteins

were separated by SDS-PAGE followed by Western blot analysis with the indicated

antibodies.

Electrophoresis and Western blot assays

Cells were cultured in 4-well plates 24 hours before performing the virus infection or

plasmid transfection. Collected cells were washed twice with 1X PBS and lysed with

lysis buffer (Cell Signaling). The protein concentrations in the cell lysates were

measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, USA) according to

the manufacturer's protocol. An equal quantity of each sample was mixed with 2.5X

Laemmli loading buffer and incubated in boiling water for 10 minutes. Following

electrophoresis on an SDS-polyacrylamide gel, the samples were transferred to a PVDF

membrane (GE Healthcare Life Sciences, Chicago, USA). The detection of protein

expression was carried out using specific primary antibodies along with HRP-labeled

secondary antibodies. X-ray films (Kodak, Rochester, USA) were used to capture the

results after the membrane was exposed to ECL Plus reagent (Amersham Biosciences,

Little Chalfont, England). Quantification of target proteins was performed using ImageJ.

Detection of LC3 puncta

To clarify the effects of ARV infection on autophagy, cells were transfected with the

LC3-GFP plasmid to observe LC3 puncta fluorescence following ARV infection. For

the experiment, vero cells were seeded on 18x18 mm coverslips. Following treatment, the cells were washed twice with 1 x PBS and then fixed at the indicated times with 4% paraformaldehyde (Alfa Aesar, Haverhill, MA, USA) for 20 min at room temperature. Subsequently, fixed cells were incubated in PBS containing 0.1% Triton X-100 for 10 min. After washing twice with 1X PBS, the cells were blocked with SUPERBLOCK®T20 solution (Thermo Scientific, Bellefonte, USA) for 1 hour at room temperature. The cells were washed twice with 1 x PBS and stained with 4', 6-diamidino-2-phenylindole (DAPI) for 10 min. After washing the cells three times with 1 x PBS, they were mounted onto glass slides using ibidi mounting medium (ibidi GmbH, Lochhamer Schlag, Germany), followed by observation with a BX51 fluorescence microscope.

Statistical analysis

All data obtained in this study were evaluated for statistical significance using the Student's t-test. The data were expressed as averages of three independent experiments. P values of less than 0.05 were considered statistically significant.

Author contribution

All authors made substantive intellectual contributions to the present study and approved the final manuscript. H.J.L. conceived of the study and generated the original hypothesis, wrote the paper, and supervised the project; W.R.H. performed most of the experiments. J.Y.L, T.L.L, L.F.L, M.M, and H.J.L. analyzed data; H.J.L. and M.M. revised and edited the manuscript.

CONFLICT OF INTEREST

We declare that we have no competing interests.

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Table 1. The catalog numbers and dilution factor of the respective antibodies used in this study

Antibodies	Catalog numbers	Clone name	Dilution factor	Manufacture
*Mouse anti-p17	-	-	2000	Our laboratory
Mouse anti-σA	-	-	4000	Our laboratory
Mouse anti-σC	-	-	4000	Our laboratory
Mouse anti-casein kinase IIα	sc-12738	1AD9	1000	Santa Cruz
Rabbit anti-CK2 Substrate [(pS/pT)DXE]	8738	-	1000	Cell Signaling
Mouse anti-IKKα	11930	3G12	2000	Cell Signaling
Rabbit anti-p-IκBα (S32)	2859	14D4	1000	Cell Signaling
Mouse anti-IκBα	4814	L35A5	2000	Cell Signaling
Rabbit anti-NF-κB p65	4764	C22B4	2000	Cell Signaling
Rabbit anti-NF-κB p50	3035	-	1000	Cell Signaling
Mouse anti-PhLP	sc-376918	A-8	1000	Santa Cruz
Rabbit anti-PFDN5	ab129116	FPR7755	2000	Abcam
Rabbit anti-CCT2	ab92746	EPR4084	3000	Abcam
Rabbit anti-CCT5	ab129016	EPR7562	3000	Abcam
Rabbit anti-HSPA8	8444	D12F2	2000	Cell Signaling
Rabbit anti-Hsp90	4877	C45G5	2000	Cell Signaling
Rabbit anti-p-Cdc37 (S13)	13248	D8P8F	2000	Cell Signaling
Mouse anti-Cdc37	sc-17758	C-11	2000	Santa Cruz
Mouse anti-ubiquitin	sc-8017	P4D1	1000	Santa Cruz
Rabbit anti-LAMP2	49067	D5C2P	2000	Cell Signaling
Rabbit anti-Beclin1	3495	D40C5	2000	Cell Signaling
Rabbit anti-LC3B	3868	D11	2000	Cell Signaling
Rabbit anti-PI3 Kinase Class III	4263	D9A5	2000	Cell Signaling
Rabbit anti-β-tubulin	2128	9F3	2000	Cell Signaling
Mouse anti-GAPDH	ab8245	6C5	3000	Abcam
Rabbit anti-Histon H2A	2578	-	2000	Cell Signaling
Mouse anti-β-actin	MAB1501	C4	10000	Millipore
Goat anti-mouse IgG (H+L) HRP	5220-0341	-	5000	SeraCare

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Goat anti-rabbit IgG (H+L) HRP	5220-0336	-	5000	SeraCare

855 *Polyclonal antibodies

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858

859 **Table 2. The primers used in this study**

Gene	Accession number	Sequence (5′-3′)	Location	Expected size (bp)
Generation of pCI-Flag-p17 and Ck2 plasmids for transfection				
p17 (full)	AF330703	F : CG<u>GAATTC</u>ATGCAATGGCTCCGCCATACGA (EcoR I)	293-314	441
		R : GC<u>TCTAGA</u>TCATAGATCGGCGTCAAATCGC (Xba I)	733-712	
Casein kinase 2	KF741763.1	F : <u>TCTAGA</u>ACGATGGCGCGTGCCATATACGACTT (Xba I)	16-38	1251
		R : <u>GTCGAC</u>CCTAGGCGGTAAAAGTGGC (Sal I)	1266-1249	
Primers for semi-quantitative RT-PCR				
Beclin1	NM_001266685	F : CATTACTTACCACAGCCCAG	134-154	321
		R : CTGAGTGTCCAGCTGGTCTA	454-433	
GAPDH	NM_002046	F : CACCACCATGGAGAAGGCTGGGGCTCA	480-506	454
		R : GGCAGGTTTCTCCAGACGGCAGGTCAG	933-907	

860

861 **Figure legends**

862 **Figure 1**. ARV regulates IκBα through TRiC chaperone. (A) Vero cells were treated

863 with TRiC inhibitor HSF-1 (40 μM) and then infected with ARV at an MOI of 10 for

864 24 h. In co-immunoprecipitation experiments, cells lysates were immunoprecipitated

865 with CCT2 antibody and analyzed by Western blot assays with the indicated antibodies.

866 (B) Vero cells were infected with ARV at an MOI of 10 for 24 h. In co-

867 immunoprecipitation experiments, cells lysates were immunoprecipitated with IκBα

868 antibody and analyzed by Western blot assays with the indicated antibodies. (C) Vero

869 cells were transfected using the shRNAs for 6 h, followed by infection with ARV at an

870 MOI of 10 for 24 h. In co-immunoprecipitation experiments, the binding of CCT2, ARV

871 σC and IκBα were examined in either ARV-infected or ARV- and CK2 shRNA or Hsc70

872 shRNA-co-transfected Vero cells., Detection of protein expression was performed by

36

873   Western blot assays with indicated antibodies. (D) To study the role of co-chaperones

874   in ARV-regulating the NF-κB signaling, Vero cells were pretreated with either TBB (5

875   μM) or VER-155008 (5 μM) for 1 hour followed by infection with ARV (10 MOI) for

876   24 hours. Whole cell lysates were immunoprecipitated with anti-σC and anti-IκBα

877   antibodies and analyzed by Western blot assays with the indicated antibodies. Co-

878   immunoprecipitation experiments of IκBα, σC, Hsc70, CCT2, and PhLP1 were

879   performed as described above. IgG was used as a negative control. (E) The binding of

880   CCT2 to σC and IκBα in ARV infected- or p17-transfected cells were examined. Cells

881   lysates were immunoprecipitated with CCT2 and detected by Western blot assays with

882   the indicated antibodies. All experiments were conducted in three independent

883   experiments. All original blots and images are shown in Fig. S

884

885   **Figure 2**. ARV p17 modulates the interaction between co-chaperones and TRiC without

886   altering their protein expression levels. To investigate whether the p17 protein

887   modulates phosphorylation of CK2, proteins levels of co-chaperones in ARV-infected

888   and p17-transfected Vero cells were analyzed. The expression levels of p17, Hsp90,

889   Cdc37, and p-Cdc37 in Vero cells infected with ARV at an MOI of 10 or transfected

890   with the pCI-Flag-p17 plasmid were examined by Western blot assays. Vero cells were

891   ARV-infected and pCI-Flag-p17-transfected at the indicated time points. The levels of

892   the indicated proteins in the mock-control group (0 h) were considered onefold. The

893   fold changes indicated below each lane were normalized against values for the mock-

894   control group. Protein levels were normalized to those for β-actin. Signals in all Western

895   blots were quantified with ImageJ software. All experiments were conducted in three

896   independent experiments.

897

898 **Figure 3**. Proximity ligation assays for CCT2 and LC3-II detection in Vero cells. (A)

899 To analyze interactions of CCT2 and LC3-II, Vero cells were detected using the

900 commercial kit Duolink (Sigma-Aldrich, cat# DUO 92008) based on *in situ* proximity

901 ligation assay according to manufacturer´s instructions. Enlarged images correspond to

902 the region indicated by the white box in the merged image. Representative images are

903 from three independent experiments. (B) To confirm ARV-mediated induction of CMA

904 through CCT2, co-immunoprecipitation was performed to analyze the interactions

905 among CCT2, LC3-II, and LAMP2. After 24 hours of ARV infection, cellular proteins

906 were collected for the co-immunoprecipitation assay. Whole cells lysates were

907 immunoprecipitated with CCT2 antibody and analyzed by Western blot assays with the

908 indicated antibodies. IgG was used as a negative control. (C) Vero cells were transfected

909 with the mCherry-Lc3 plasmid for 6 h, followed by infection with ARV at an MOI of

910 10 for 24 h. Vero cells were fixed and processed for immunofluorescence staining with

911 DAPI (blue). Stained Lc3 (red) and CCT2 (green) were observed under a fluorescence

912 microscope.

913

914 **Figure 4**. The p17 protein of ARV acts as a NF-κB activator. (A) The levels of p-IκB,

915 IκB, p65, and p50 in mock control, pCI-Flag vector, ARV-infected, and pCI-Flag-p17-

916 transfected cells were examined. Cytoplasmic and nuclear protein extractions were

917 carried out using the Compartmental Protein Extraction. The cytoplasmic extracts and

918 nuclear extracts were detected at the indicated time points for Western blot assays. The

919 protein levels were normalized to that for β-actin or histone 2A. Numbers below each

920 lane are percentages of the control level of a specific protein in the mock treatment.

921

922 **Figure 5**. The p17 protein of ARV protects IKK from polyubiquitination and enhances

923   IκB degradation. (A) Vero cells were transfected with CK2 shRNA for 6 h, followed by

924   infection with ARV at an MOI of 10 for 24 h. The binding of Hsp90 to Cdc37 and IKK

925   in ARV infected- or p17-transfected cells were examined in the present or absence of

926   CK2 shRNA. Cells lysates were immunoprecipitated with the Hsp90 antibodies and

927   detected with the indicated antibodies. (B) To investigate whether ARV p17 induced

928   Hsp90 protects IKK or IκB proteins from ubiquitin-proteasome-mediated degradation,

929   Vero cells were transfected with the pCI-Flag-p17 for 6 hours, followed by treatment

930   of 17-AAG (5 μM) for 24 hours. Cells lysates were immunoprecipitated with IKK or

931   IκB antibodies, and the interaction of ubiquitin with IKK and IκB were examined. The

932   amounts of IKK and IκB polyubiquitylation were examined by Western blot assays.

933   Rabbit IgG served as negative control. Similar results were obtained in three

934   independent experiments.

935

936   **Figure 6**. ARV p17-induced autophagy through the Hsp90/NF-kB pathway. (A) The

937   expression level of p65 and p50 in ARV-infected and p17-transfected cells were

938   examined in the present or absence of TBB (5 μM). Nuclear extracts from ARV-infected

939   and mock-infected Vero cells were collected for Western blots. The protein levels were

940   normalized to that for histone 2A. The levels of indicated proteins at mock were

941   considered 1-fold. (B) To study the role of Hsp90 in p17-regulating the NF-κB pathway,

942   Vero cells were pretreated with either 17-AAG (5 μM) or Bay11-7085 (5 μM) for 1

943   hour followed by infection with ARV (10 MOI) or transfection with pCI-Flag-p17 for

944   24 hours. The cytoplasmic lysates and nuclear extracts were collected for Western blots.

945   The levels of indicated proteins in the mock control or at 0 h were considered 1-fold.

946   (C) ARV stimulates the Beclin1 transcription through the NF-κB pathway. Upper panel:

947   The mRNA levels of Beclin1 in the Vero cells were examined by semi-quantitative RT-

948　PCR in ARV-infected and mock-infected cells in the presence or absence the different

949　concentrations of Bay11-7085. After electrophoretic separation in an agarose gel, PCR

950　products were stained with ethidium bromide. Graph shown represents the mean± SD

951　calculated from three independent experiments. Lower panel: The expression level of

952　Beclin1 in ARV-infected and mock-infected cells were examined in the present or

953　absence of Bay11-7085. Whole cell lysates were harvested for Western blots.

954

955　**Figure 7**. The p17　protein of ARV enhances the PtdIns3K/ Beclin1 complex leading

956　to induction of autophagosome formation. (A) To confirm whether CK2 is the upstream

957　signaling that regulates Cdc37, Beclin1, and LC3, the proteins levels were examined in

958　CK2 knockdown and CK2 overexpression in p17-transfected Vero cells. The protein

959　levels were normalized to that for β-actin. The activation and inactivation folds

960　indicated below each lane were normalized against mock control. (B) Vero cells were

961　pretreated with either 17-AAG (5 μM) or Bay11-7085 (5 μM) for 1 hour followed by

962　infection with ARV (10 MOI) or transfection with pCI-Flag-p17 for 24 hours. Whole

963　cell lysates were collected for Western blots with the indicated antibodies. The levels

964　of indicated proteins at mock were considered 1-fold. (C) ARV p17 facilitates

965　autophagosome formation by promoting the PtdIns3K/Beclin1 complex via the

966　CK2/Hsp90 pathway. In reciprocal co-immunoprecipitation experiments, the binding

967　of PtdIns3K and Beclin1 was examined in either p17-transfected or p17 and Hsp90 or

968　CK2 shRNA-cotransfected Vero cells, and detected with the indicated antibody. The

969　activation and inactivation folds indicated below each lane were normalized against

970　that at mock control.

971

972　**Figure 8**. ARV p17 promotes autophagosome formation. (A) Vero cells were

973   transfected with LC3-GFP plasmid and co-transfected with Hsp90 or CK2 shRNAs for

974   6 hours and followed with the infection of ARV an MOI of 10 for 18 h or pCI-Flag-p17

975   transfection for 24h. Cells were then fixed and processed for immunofluorescence

976   staining of DAPI. The LC3-GFP were observed under a fluorescence microscope.

977   Thapsigarigin (TG) were pretreated 2 hours before LC3-GFP transfection (positive

978   controls). (B) LC3-GFP plasmid was applied to observe LC3 punta under a

979   fluorescence microscope. Quantitation results from Fig. 8A represents mean LC3-GFP

980   puncta per cell (n=17).

981

982   **Figure 9**. ARV p17-induced autophagy through the Hsp90/NF-kB pathway in A549

983   cancer cells. (A) A549 cells were transfected with the CK2 or Hsc70 shRNAs for 6 h,

984   followed by infection with ARV at an MOI of 10 for 24 h. In co-immunoprecipitation

985   experiments, the binding of CCT2, ARV σC and IκBα were examined detected by

986   Western blot assays with the indicated antibodies. (B) A549 cells were infected with

987   ARV at an MOI of 10 for 24 h. Cell lysates were collected for immunoprecipitation

988   with the Hsc70 antibody and analyzed by Western blot assays with the indicated

989   antibodies. (C) The binding of Hsp90 to Cdc37 and IKK in ARV infected- or p17-

990   transfected cells were examined in the present or absence of CK2 shRNA. Cells lysates

991   was immunoprecipitated with Hsp90 and detected with the indicated antibodies. (D) In

992   co-immunoprecipitation experiment, the binding of class III PI3k was examined in

993   either p17-transfected or p17- and Hsp90 shRNA or CK2 shRNA-cotransfected A549

994   cells, and detected with the indicated antibody. The activation and inactivation folds

995   indicated below each lane were normalized against that at mock control. (E) A549 cells

996   were transfected with LC3-GFP plasmid and co-transfected with Hsp90 or CK2

997   shRNAs for 6 hours, and followed with the infection of ARV with an MOI of 10 for 18

998   h or pCI-Flag-p17 transfection for 24h. Cells were then fixed and processed for

999   immunofluorescence staining of DAPI. The LC3-GFP were observed under a

1000   fluorescence microscope. Each experiment was performed at least three times. (F) The

1001   numbers of LC3 punta were calculated from the results from Fig. 9E. Each value

1002   represents mean± SE of three independent experiments.

1003

1004   **Figure 10**. ARV modulates the IKK/NF-κB pathway via the TRiC and Hsp90

1005   chaperones, promoting chaperone-mediated autophagy benefiting viral replication. A

1006   model is proposed to illustrate that ARV p17 regulates the IKK/NF-κB pathway by

1007   modulating TRiC-co-chaperones interactions and CK2/Hsp90/Cdc37 signaling,

1008   thereby activating chaperone-mediated autophagy to enhance viral replication. Our

1009   previous study suggested that p17 mediates phosphorylation of Cdc37 through CK2

1010   [50]. Phosphorylation of PhLP1 is mediated by the protein kinase CK2 [52]. The figure

1011   was created with BioRender.com.