1 **The p7 protein of the hepatitis C virus induces cell death differently from the influenza**

2 **A virus viroporin M2.**

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18   **Abstract**

19   Most viruses encode proteins that modulate cell-death signaling by the host. For hepatitis C

20   virus (HCV) infection, apoptosis and other forms of cell-death have been observed in vitro

21   and in vivo but the detailed understanding of this intricate viral-host interplay is unclear. This

22   study examined the role played by the HCV p7 protein in the induction of cell-death. By

23   measuring caspase-3/7 activation and cleavage of endogenous PARP, two hallmarks of

24   apoptosis, the overexpression of p7 protein was shown to induce apoptosis in Huh7.5 cells.

25   Furthermore, p7-induced apoptosis is caspase-dependent and involves both the intrinsic and

26   extrinsic pathways. Similar to the M2 protein of influenza A virus, p7-induced apoptosis is

27   independent of its ion channel activity. Coimmunoprecipitation experiments further showed

28   that both M2 and p7 interact with the essential autophagy protein Beclin-1. However, only

29   the M2 protein could cause an increase in the level of LC3-II, which is an indicator of

30   autophagic activity. Thus, although the p7 protein is functionally similar to the well-

31   characterized M2 protein, they differ in their activation of autophagic cell-death. Taken

32   together, these results shed more light on the relationship between the HCV p7 ion channel

33   protein and cell-death induction in host cells.

34

35   **Keywords**: HCV, p7 protein, cell death, ion channel activity

36

1. Introduction

Hepatitis C virus (HCV), a small positive strand RNA virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family, is one of the major causes of liver disease such as cirrhosis, steatosis, and hepatocellular carcinoma (Chen and Morgan, 2006). In fact, HCV infection is the most frequent indication for liver transplantation in developed countries (Brown, 2005). Currently, about 130 - 170 million or 3 % of the world population are estimated to be infected with HCV; with about 3 to 4 million new infections per year (WHO, 2012). HCV has a high degree of genetic diversity, so it is classified phylogenetically into six major genotypes and several subtypes (Simmonds, 2004). The HCV genome of ~9.6 kb encodes a unique open reading frame that is translated into a precursor polyprotein of ~3000 residues. Co- and post-translational processing of the polyprotein by both viral and cellular proteases yields three structural (Core, E1 and E2), six non-structural proteins (NS2-NS5), and a small membrane ion channel protein (p7) (Lin et al., 1994); the protein of interest in this study.

The HCV p7 protein, a small (63 amino acid) hydrophobic protein located between the structural and nonstructural region of HCV, is not clearly identified as structural or nonstructural protein, although it has been shown to be a transmembrane protein (reviewed in (Khaliq et al., 2011)). HCV p7 is classified as a viroporin, a group of small hydrophobic proteins encoded by a variety of RNA viruses that oligomerize to form pores (ion channels) in host-cell membranes through which viruses can enter/exit as well as contribute to virus assembly and pathology of disease by altering membrane permeability and disrupting ion homeostasis in cells (Gonzalez and Carrasco, 2003). The precise role of p7 in the HCV life-cycle has been hard to define. However, some recent papers have implicated p7 in the assembly and release of virus particles, mostly acting in concert with other viral proteins (reviewed in (Khaliq et al., 2011; Steinmann and Pietschmann, 2010)), and in a genotype-

62 specific manner (Steinmann et al., 2007). For example, a strain-specific tripartite relationship

63 between core, p7 and NS2 has been reported to be responsible for modulating the subcellular

64 localization of core (Boson et al., 2011) and NS2 (Tedbury et al., 2011), which is independent

65 of p7's ion channel activity. Similarly, it has been shown that mutations in the p7 protein,

66 either singly or in combination with E2 glycoprotein enhances several-fold production of

67 infectious virus particles in cell culture (Kim et al., 2011). Thus, p7 unlike other viroporins

68 such as M2 of influenza A virus and Vpu of HIV-1, is absolutely essential for HCV

69 replication in vitro (Brohm et al., 2009; Steinmann et al., 2007).

70   Cell death regulation is an important determinant in the survival of most viruses,

71 therefore, many viruses encode proteins that interfere with cell death signaling pathways,

72 skewing it in their favour (Chen et al., 2006). For HCV, both proapoptotic and prosurvival

73 properties have been attributed to different HCV proteins (reviewed in (Aweya and Tan, 2011;

74 Fischer et al., 2007)). HCV p7 like other members of the viroporins (e.g., HIV-1 Vpu, human

75 T-cell lymphotropic virus-1 p13II, hepatitis B virus X protein, and influenza virus PB1

76 ORF2), might be targeted to the mitochondrial membranes where it modulates apoptosis by

77 altering the mitochondrial membrane permeability. However, there is currently limited

78 information on the apoptotic activity of p7 except that reported by Madan *et al* (Madan et al.,

79 2008) who demonstrated that the p7 protein of genotype 1b HCV induces caspase-dependent

80 apoptosis via the mitochondria in baby hamster kidney cells. Since the genotype 2a JFH1

81 strain is the only one that can replicate efficiently in permissive cells, such as Huh7.5 cells,

82 without adaption, this study sought to examine how p7 protein of this strain modulates cell-

83 death and how similar it is to the M2 ion channel protein of influenza A virus, which is a well

84 characterized viroporin. We were able to show that HCV p7 protein induces caspase-

85 dependent apoptosis which is independent of its ion channel activity, and although p7 protein

86   shares a couple of functional properties with M2 protein of influenza A virus, they seem to

87   differ in their induction of autophagic cell death.

88

89

90 **2. Materials and Methods**

91 *2.1. Cell culture and cell lines*

92   Huh7.5 cells (subclone of the Huh-7 human hepatoma cell line; Apath, Brooklyn,

93 NY) and 293FT cells (human embryonic kidney cell line with the temperature sensitive gene

94 for SV40 T-antigen; Invitrogen, Karlsruhe, Germany) were grown in Dulbecco's modified

95 Eagle's medium (Invitrogen) supplemented with 10 % fetal bovine serum (HyClone, Utah,

96 USA), nonessential amino acids and antibiotics (10 units/ml penicillin and 10 μg/ml

97 streptomycin) (Invitrogen, Carlsbad, CA). All cells were maintained in a 37 °C incubator

98 with 5 % CO_2.

99

100 *2.2. Plasmid Construction*

101   Expression plasmids for the HCV p7 proteins were generated by PCR cloning using

102 Titanium Taq DNA polymerase (Clontech Laboratories Inc., Palo Alto, CA). Two plasmids

103 containing the full-length HCV genome of the genotype 1b (S1) cloned in Singapore ((Soo et

104 al., 2002) GenBank accession number AF356827) and genotype 2a JFH-1 strain (Wakita et

105 al., 2005), GenBank accession number AB047639) were used as templates. The PCR

106 products were digested with restriction enzymes BamHI and XhoI and then ligated into the

107 pXJ40flag vector which is a flag-tagged plasmid derived from pXJ40 (Xiao et al., 1991). The

108 pXJ40flag vector was used so that a flag epitope is fused to the N-terminus of the p7 protein

109 to allow the comparison of protein expression levels with an anti-flag antibody. Similarly, the

110 p7 mutants were generated by 2 rounds of PCR: the first round of PCR was used to generate

111 5' and 3' fragments of p7 containing the appropriate alanine or glutamine substitutions; these

112 were then amplified into full-length p7 using end primers for the p7 gene and cloned into

113 pXJ40flag vector using BamHI and XhoI sites. The M2 gene of influenza A virus (A/Puerto

114 Rico/8/34/Mount Sinai/Wi (H1N1), GenBank accession number AY768951.1) was produced

115 by gene synthesis (GenScript USA Inc Piscataway, NJ, USA) and cloned into the pXJ40flag

116 vector in a similar manner. All sequences were confirmed by sequencing performed by the

117 DNA Sequencing Facility at the Department of Microbiology, Yong Loo Lin School of

118 Medicine, National University of Singapore, Singapore.

119

120 *2.3. Transient transfections and Western blot analysis*

121 Transient transfections of cells were performed using Lipofectamine 2000 reagent

122 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Approximately 24 h

123 after transfection, the cells were harvested by scrapping into the media, spun down in a

124 bench-top centrifuge and washed twice with cold PBS. The cell pellets were then

125 resuspended in RIPA buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.5 % NP40, 0.5 %

126 deoxycholic acid, 0.005 % SDS and 1 mM phenylmethylsulfonyl fluoride) and subjected to

127 freeze-thawing five times before being spun down at 13000 rpm to remove cellular debris.

128 The cell lysate was then used for Western blot analysis and quantification of apoptosis (see

129 next section below).

130 Primary antibodies used in the study included anti-actin monoclonal, anti-flag

131 monoclonal and polyclonal (Sigma, St. Louis, MO), anti-poly [ADP-ribose] polymerase

132 [PARP] polyclonal, and anti-LC3B polyclonal (Cell Signaling Technology Inc., Beverly, MA)

133 antibodies. Secondary antibodies used included horseradish peroxidase (HRP)-conjugated

134 goat anti-mouse, and goat anti-rabbit antibodies (Pierce, Rockford, IL).

135

136 *2.4. Apo-One fluorometric and TUNEL assay*

137 Apoptosis was quantified in the form of caspase-3/7 activation using the Apo-One

138 fluorometric assay system from Promega Corporation (Madison, WI) according to the

139 manufacturer's protocol. To further confirm the induction of apoptosis by cells

140   overexpressing p7 protein, the Terminal deoxynucleotidyl transferase dUTP nick end labeling

141   (TUNEL) assay was carried out after 24 h of transfection using the DeadEnd Fluorometric

142   TUNEL system (Promega, Madison, WI) according to the manufacturer's protocol. Briefly,

143   cells were plated in Lab-Tek™ Chamber Slides, transfected with plasmids expressing p7

144   protein followed by treatment with cell permeable caspase inhibitors as described below.

145   After 24 h, cells were fixed in 4% paraformaldehyde at 4°C for 25 min. Fixed cells were then

146   permeabilized in 0.2% Triton X-100 and labelled with fluorescein-12-dUTP using terminal

147   deoxynucleotidyl transferase. After rinsing with PBS, slides were mounted with

148   VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA).

149   The TUNEL-positive cells (green fluorescence) corresponding to the nuclei location (DAPI)

150   were captured with Olympus FluoView FV1000 (Olympus, Japan) laser scanning confocal

151   microscope using a 60x/1.45 oil objective, with 543nm HeNe laser as the excitation source.

152

153   *2.5. Treatment of cells with caspase inhibitors and ammonium chloride*

154   Cells were transfected (as above) followed by treatment with 2.5 μM of caspase-3 (Z-

155   DEVD-fmk), caspase-8 (Z-IETD-fmk), caspase-9 (Z-LEHD-fmk) or a negative caspase

156   inhibitor (Z-FA-fmk) (BD Biosciences). Briefly, 6 h post-transfection, the transfection media

157   was changed and replinished with fresh media plus 2.5 μM of the respective cell permeable

158   caspase inhibitors which are reported to have a very short half-life (Ekert et al., 1999; Garcia-

159   Calvo et al., 1998). After about 24 h post-transfection, the cells were harvested by scrapping

160   into the media, spun down in a bench-top centrifuge and washed three times with 1X cold

161   PBS to remove any residual inhibitor found in the media. The cell pellets were then

162   resuspended in RIPA buffer and subjected to freeze-thawing five times before being spun

163   down at 13000 rpm to clarify the lysate. Subsequently, the lysate was analysed using the

164   Apo-One fluorometric assay as described above.

165   In order to block autophagosome formation, cells were treated with ammonium

166   chloride (BDH, AnalaR). Briefly, the spent media from an overnight transient transfection

167   was replaced with complete media containing 2.7 mg/ml of ammonium chloride and then

168   incubated further for 2.5 h. Cells were then washed twice with 1X cold PBS, lysed directly in

169   2X Laemmli's sodium dodecyl sulfate (SDS) buffer and the cell lysate subjected to SDS-

170   polyacrylamide gel electrophoresis, followed by Western blot analysis to determine the

171   conversion of LC3-I to LC3-II.

172

173   *2.6. Coimmunoprecipitation experiments*

174   For the coimmunoprecipitation experiments, each 6 cm dish of cells was resuspended

175   in 400 µl of RIPA buffer and subjected to freeze-thawing five times. Anti-Beclin-1 rabbit

176   polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the lysates

177   containing 200 µg of total protein and allowed to mix for 1 h at 4 °C. Protein A agarose beads

178   (Roche, Indianapolis, IN) were added, and the mixture was subjected to end-over-end mixing

179   at 4 °C overnight. Beads were washed four times with cold RIPA buffer, and then 15 µl of

180   Laemmli's SDS buffer was added and the samples were boiled at 100 °C for 5 min to release

181   the immunocomplexes. Samples were separated by SDS-polyacrylamide gel electrophoresis

182   and subjected to Western blot analysis as described above.

183

184   *2.7. Membrane permeabilization assay*

185   Permeability of the plasma membrane of cells expressing p7 or mutants to

186   hygromycin B (a translation inhibitor) was a modification of the method described by Liao *et*

187   *al* (Liao et al., 2006). Briefly, 293FT cells in 6 cm dishes were transfected with plasmids as

188   described above, followed by treatment of the cells with different concentrations of

189   hygromycin B (Roche) for 30 min at 6 h post-transfection. After 24 h post-transfection, the

190 cells were harvested, lysed in RIPA buffer and subjected to freeze-thaw five times. The cell

191 extracts were then spun at 13,000 rpm for 20 min at 4 °C to clarify the lysate. The proteins

192 were immunoprecipitated with anti-Flag beads (Sigma) overnight at 4 °C, and washed three

193 times with RIPA buffer. The samples were then analyzed by 15% SDS-PAGE.

194

195 *2.8. Immunofluorescence assay*

196 For indirect immunofluorescence staining, transfected Huh 7.5 cells grown on coverslips

197 were fixed with 4 % paraformaldehyde in PBS for 15 min. Fixed cells were permeabilized

198 with 0.2% Triton X-100 in PBS for 5 min, blocked with 1 % bovine serum albumin (BSA) in

199 PBS for 30 min and incubated with primary antibodies (anti-flag polyclonal (Sigma)) for 1 h.

200 After washing, cells were incubated with Alexa-Fluor-564-conjugated goat anti-rabbit IgG

201 secondary antibodies (Invitrogen) for 1 h. Images were captured with Olympus BX60

202 fluorescence microscope (Olympus, Japan) using a 100x oil objective.

203

204 *2.9. Quantification of autoradiographs and statistical analysis*

205 ImageJ open-source gel analysis software (Rasband) was used for the quantification

206 of the intensities of specific bands on autoradiographs. All experiments were repeated at least

207 three times. The mean data is presented. Statistical analysis was performed using the

208 Student's t-test. $p < 0.05$ was considered significant.

209

3. Results

3.1. HCV p7 protein induces apoptosis in Huh7.5 cells

To determine if genotype 2a HCV p7 protein can induce apoptosis in host cells, Huh7.5 cells, which are highly permissive for HCV replication, were transiently transfected with a cDNA expression plasmid containing the genotype 2a (JFH-1 strain) p7. For comparison, Huh7.5 cells were also transfected with genotype 1b (S1) HCV p7 protein, the empty vector or an expression plasmid containing the Bax gene, which is a well-known apoptosis inducer that acts via the mitochondria. About 24 h post-transfection, the cells were harvested, lysed and the cell lysate was used for western blot analysis and measurement of the activation of caspase-3/7, which is a hallmark of apoptosis. As shown in Fig.1A, cells overexpressing genotype 1b p7 (abbreviated p7-1b), genotype 2a p7 (abbreviated p7-2a), or Bax have significantly higher level of caspase-3/7 activity than the vector control cells. Similarly, the cleavage of endogenous poly (ADP-ribose) polymerase (PARP), a substrate of activated caspase-3/7, was clearly observed in cells overexpressing p7-1b, p7-2a and Bax but there was less cleavage in cells transfected with the empty vector (Fig. 1B, middle). Western blot analysis showed the expression of flag-tagged protein (Fig. 1B, top) and endogenous actin (Fig. 1B, bottom). The rest of the experiments were performed using only the genotype 2a p7 (hereafter p7).

3.2. HCV p7-induced apoptosis is caspase-dependent

To determine if p7-induced apoptosis is caspase-dependent, Huh7.5 cells were transiently transfected with the p7 plasmid, followed by treatment with cell permeable pan-caspase inhibitor (zVAD-fmk) or an irrelevant peptide (zFA-fmk) 6 h post-transfection. The level of apoptosis induced, as measured by caspase-3/7 activity, was observed to be significantly inhibited by the pan-caspase inhibitor but not the irrelevant peptide, suggesting

235 the involvement of a caspase-dependent pathway in p7-induced apoptosis (Fig. 2A).

236 Consistently, there was only a low level of cleaved PARP, as detected by western blot

237 analysis, in the p7-overexpressing cells treated with zVAD-fmk treated cells (Fig. 2B,

238 middle). However, a high level of cleaved PARP was observed in the p7-overexpressing

239 cells that were treated with zFA-fmk (Fig. 2A, densitometry values below). To confirm the

240 involvement of caspases in p7-induced apoptosis, the terminal uridine nick 3′ end-labeling

241 (TUNEL) assay was performed. Cells overexpressing p7 and treated with the irrelevant

242 peptide (zFA-fmk) had more TUNEL-positive cells than those treated with the pan-caspase

243 inhibitor (zVAD-fmk) (Fig. 4, top right panel).

244

245 *3.3. HCV p7 protein induces apoptosis via both the intrinsic and extrinsic pathways*

246 Since p7 induces apoptosis via caspase-dependent pathway, the next step was to

247 determine which of the caspase-dependent pathways are involved. To examine this, cell

248 permeable non-toxic peptide caspase inhibitors that bind irreversibly to activated caspases

249 and which have a short half-life (Ekert et al., 1999; Garcia-Calvo et al., 1998) and specific for

250 different caspases, namely caspase-3 (zDEVD-fmk), caspase-8 (zIETD-fmk) and caspase-9

251 (zLEHD-fmk), were added to the cells 6 h post-transfection. As shown in Fig. 3A, the level

252 of p7-induced apoptosis after about 24 h post-transfection, as reflected by the caspase-3/7

253 activity in the cell lysate, was significantly inhibited by all three caspase inhibitors, but not by

254 the irrelevant peptide (zFA-fmk). However, high levels of cleaved PARP were still observed

255 in cells treated with the caspase inhibitors despite the low caspase-3/7 activity (Fig. 3B,

256 middle). This could possibly be due to the cleavage of PARP by caspase-independent events

257 (Yang et al., 2004) and other caspases in the absence of caspase-3 or caspase-7 activation.

258 Indeed, it has been reported that PARP can be cleaved by other proteases and protease-like

259 molecules as well as several caspases (Fernandes-Alnemri et al., 1995; Germain et al., 1999;

260 Lippke et al., 1996; Malireddi et al., 2010; Muzio et al., 1996; Orth et al., 1996; Tewari et al.,

261 1995) albeit at lower efficiency than by caspase-3 or -7. Similarly, the number of TUNEL-

262 positive cells was not much different between cells treated with the irrelevant peptide (zFA-

263 fmk) and the three caspase inhibitors (Fig. 4, lower panel). One could therefore speculate that,

264 probably because p7-induced PARP cleavage and DNA fragmentation are late apoptosis

265 events (Collins et al., 1997; Duriez and Shah, 1997), they could not be completely blocked

266 when a caspase inhibitor that is specific for only one of the caspases is used. Nevertheless,

267 our results showed that p7-induced apoptosis is indeed caspase-dependent and involves

268 activation of both caspase-8 and -9 which are initiator caspases of the death receptor

269 (extrinsic) and mitochondria-mediated (intrinsic) pathways respectively, and found upstream

270 of caspase-3/7, the executioner caspases.

271

272 *3.4. HCV p7-induced apoptosis does not depend on its ion channel activity*

273 The HCV p7 protein possesses ion channel activity and is thus classified in the family

274 of viral ion channel proteins known as viroporins (Griffin et al., 2003; Pavlovic et al., 2003).

275 Moreover, H17, located within the amino-terminal transmembrane domain of p7, has been

276 implicated in p7's ion channel function, and has also been shown to share functional

277 similarity with H37 of the influenza A virus M2 ion channel protein (StGelais et al., 2009).

278 However, the p7 of genotype 2a has an additional H31, which is found in only a few other

279 genotypes and thought to partly influence the gating function of p7 (Brohm et al., 2009;

280 Montserret et al., 2010). Thus, in order to express p7 lacking ion channel activity and to

281 eliminate the probable influence of H31, both H17 and H31 were substituted with A to

282 generate a double substitution mutant p7H17AH31A. Using the membrane permeabilization

283 assay (see materials and methods section for details), the overexpression of p7 resulted in the

284 permeabilization of the cell membrane, as the translation of p7 protein was clearly inhibited

285   in the presence of Hygromycin B (Fig. 5A, left panel). In contrast, the overexpression of

286   p7H17AH31A seems to have little effect on membrane permeability as the addition of

287   Hygromycin B did not inhibit the translation of p7H17AH31A to a great extent (Fig. 5A,

288   right panel).

289   When the level of apoptosis of cells overexpressing p7 and the double mutant was

290   subsequently measured, it was observed that both the wild-type p7 protein and the

291   p7H17AH31A mutant induced significantly higher caspase-3/7 activity than the vector (Fig.

292   5B). It therefore appears that p7-induced apoptosis is independent of its ion channel activity.

293   In addition, the cleavage of endogenous PARP, a substrate of activated caspase-3/7, was

294   clearly observed in Huh7.5 cells overexpressing either the wild-type p7 protein or the

295   p7H17AH31A mutant (Fig. 5C, middle).

296

297   *3.5. Mutation of the conserved dibasic amino acids in the cytoplasmic domain of p7 protein*

298   *affects its apoptosis activity*

299   Apart from the histidine residues in p7, there are two highly conserved basic amino

300   acids (K/R33 and R35) in the cytoplasmic domain of p7 protein. When these dibasic residues

301   in p7 are substituted by A or Q, the ion channel activity of p7 is lost and the production of

302   infectious particles is reduced (Griffin et al., 2004; Steinmann et al., 2007). To determine if

303   these amino acid residues are essential for the apoptotic activity of p7 protein, substitution

304   mutants were generated in the background of genotype 2a HCV p7 protein. Using the

305   membrane permeabilization assay, we demonstrated that these mutants lacked ion channel

306   activity (results not shown). However, compared to the wild-type p7 protein, overexpression

307   of the mutants had a significantly lower level of caspase-3/7 activity (Fig. 6A). Both single

308   and double substitution mutants were expressed at higher levels than the wild-type p7 protein,

309   indicating that the lack of apoptosis is not a consequence of lower protein expression levels

310   (Fig. 6B, top). As it has been reported that these residues (R33 and R35) are important in

311   maintaining the topology of p7 (Steinmann et al., 2007), the mutants could not induce much

312   apoptosis as the wild-type p7 probably because they are orientated wrongly in the host

313   membranes.

314

315   *3.6. HCV p7 protein does not induce autophagic cell death but can interact with Beclin-1*

316   Based on the above characterization of p7, it seems that p7, like M2 protein, can

317   induce caspase-dependent apoptosis, but this is independent of ion channel activity. M2 has

318   also been shown to block autophagosome fusion with lysosomes and resulting in cell death

319   (Gannage et al., 2009). Thus, to examine if p7 protein behaves in the same manner as M2,

320   Huh7.5 cells were transfected with plasmids for expressing p7 and M2 and their effects on

321   the conversion of microtubule-associated protein 1 light chain 3 (LC3) from isomer I to

322   isomer II (LC3-I to LC3-II) was analysed using western blot. However, M2 failed to block

323   macroautophagy probably due to the low transfection efficiency in Huh7.5 cells (data not

324   shown). Hence, the experiment was repeated using the highly transfectable 293FT cells

325   (Invitrogen) which is derived from human embryonal kidney cells transformed with the SV40

326   large T antigen to allow very high levels of protein expression from vectors containing the

327   SV40 origin (http://products.invitrogen.com/ivgn/product/R70007). The results showed that

328   both p7 and M2 induce apoptosis in 293FT cells when overexpressed (Fig. 7A and B), which

329   is consistent with p7 induction of apoptosis in Huh7.5 cells (see above) and M2 in BHK cells

330   (Madan et al., 2008). Interestingly, the levels of activated caspase-3/7 as well as cleaved

331   PARP were much higher in the cells over-expressing p7 compared to those over-expressing

332   M2. In lane 2, the major band for M2 is ~ 20 kDa, which is the expected molecular weight,

333   but there are also some higher molecular weight bands. These are likely to be oligomers of

334   M2 which are resistant to boiling, 20 mM dithiothreitol, and 1% SDS (which are found in the

335   Laemmli SDS loading buffer) as we have previously observed the same phenomenon with

336   another transmembrane protein (Tan et al., 2007).

337   Consistent with the previous study (Gannage et al., 2009), the level of LC3-II in M2

338   overexpressing cells was much higher than cells transfected with the empty vector (Fig. 8A

339   (middle), lanes 2 and 4). After performing four independent experiments and using

340   densitometry to quantify the intensities of LC3-I and II, the conversion to LC3-II (i.e. LC3-

341   II/LC3-I+LC3-II) was found to be significantly higher (63%) in the cells overexpressing M2

342   as compared to the cells transfected with the empty vector (49%). In contrast, the level of

343   LC3-II (the autophagic marker) in p7 overexpressing cells (41%) was not significantly

344   different from the cells transfected with the empty vector (Fig. 8B). Untransfected cells

345   untreated and treated with ammonium chloride, which is an inhibitor of autophagosome

346   formation, served as negative and positive controls respectively. As the percentage of

347   conversion to LC-II in the M2 overexpressing cells (63%) was statistically significant as in

348   the case of the ammonium chloride-treated cells (87%), the assay was deemed sensitive

349   enough to detect autophagic cell death. The expression of flag-tagged protein was detected by

350   Western blot analysis (Fig. 8A, top) and the level of endogenous actin (Fig. 8A, bottom) was

351   used as the loading control of the total proteins in the cell lysates. In addition to LC3-II as an

352   autophagosome marker, we also analyzed the accumulation of GFP-LC3 puncta (Bampton et

353   al., 2005; Mizushima, 2009). Huh7.5 cells were transiently transfected with GFP-LC3 and

354   M2 or p7 expression plasmids. Twenty-four hours post transfection; we observed that only

355   cells overexpressing M2 and GFP-LC3 showed an increased accumulation of LC3 puncta by

356   immunofluorescence microscopy analysis (Fig. 9A).

357   To further examine the difference in the ability of p7 and M2 to induce autophagic

358   cell death, co-immunoprecipitation experiment was next performed to determine if they can

359   interact with Beclin-1, which is a key component of the molecular machinery of

360   macroautophagy (reviewed in (Kang et al., 2011)) and also constitutes a major target for

361   manipulation of autophagy by viruses (Dai et al., 2012; Munz, 2011). As shown in in the top

362   panel of Figure 9B, both flag-M2 and flag-p7, but not by an irrelevant protein, glutathione *S*-

363   transferase (GST), bound to the endogenous Beclin-1 protein. The expression levels of the

364   flag-tagged proteins in the cell lysates were similar (Fig. 9B, middle) and endogenous Beclin-

365   1 expression was not affected by the expression of the flagged tag proteins (Fig. 9B, bottom).

366   Hence, it appears that the binding of p7 to Beclin-1 is not sufficient to interfere with the

367   autophagic process.

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370

4. Discussion

Currently, apoptosis or caspase-dependent cell death is the most well characterized cell death pathway, although there is increasing evidence that other non-apoptotic or caspase-independent cell death pathways are involved in programmed cell death (Hetz et al., 2005). Autophagic cell death and programmed necrosis (necroptosis) are cell death mechanisms which are also important in viral infections and are thought to be exploited by viruses for their survival (Edinger and Thompson, 2004; Park et al., 2012). There is also some crosstalk or interrelationship between the different cell death mechanisms which is exploited by some viruses for their survival in the host, as reported for influenza A virus which determines the death of its host by inducing apoptosis and blocking autophagy (Gannage et al., 2009). With some recent studies suggesting that HCV induces caspase-independent cell death such as autophagy (reviewed in (Dreux and Chisari, 2011)), it is possible that HCV, like influenza A virus, might also be exploiting the interrelationship between apoptosis and non-apoptotic cell death for its survival in the host. Incidentally, the protein of interest in this study, p7 protein, happens to share some functional relationship with influenza A virus M2 protein (Mihm et al., 2006), so it is tempting to speculate that p7 and M2 might play similar roles in cell death modulation.

Currently, the apoptotic activity of p7 of different HCV genotypes have not been reported except that of genotype 1b (Madan et al., 2008). Since the genotype 2a JFH1 strain is the only one that can replicate efficiently in permissive cells, such as Huh7.5 cells, without adaption, this study focuses on characterizing the cell death pathways activated by the p7 protein of the JFH-1 strain. To our knowledge, this is the first time that the p7 protein of genotype 2a has been shown to induce apoptosis to a similar level as that of genotype 1b p7 (Fig. 1). As for genotype 1b p7, the result is similar to the observation by Madan *et al* (Madan et al., 2008b) except that Huh7.5 cells were used in this study while BHK cells were

396   used in the previous study. Further characterization of the apoptosis induced by the p7 protein

397   of genotype 2a reveals that p7-induced apoptosis was efficiently blocked by a pan-caspase

398   inhibitor as well as inhibitors specific for caspases 3, 8 or 9 (Fig. 2 and 3). In mammalian

399   cells, apoptosis can be induced via two major pathways; the death receptor pathway (extrinsic

400   pathway), triggered by the binding of FasL to Fas (CD95), with caspase-8 as the initiator

401   caspase; and the mitochondria-mediated pathway (intrinsic pathway), induced by the

402   mitochondria in response to DNA damage, oxidative stress and viral proteins, with caspase-9

403   as the initiator caspase(Kumar, 2007). Thus, our results confirm that p7-induced apoptosis is

404   indeed caspase-dependent and involves both the intrinsic and extrinsic apoptosis pathways.

405   Given that some previous studies have reported that ions channels play a key role in

406   the regulation of cell physiology and in the induction of apoptotic cell death (Burg et al.,

407   2006; Lang et al., 2003; Szabo et al., 2004), it was important to also determine whether this

408   property of p7 has a link with its apoptotic activity. Although, details and the exact function

409   of most ion channels in apoptosis mediation are currently unknown, it is believed that some

410   viruses employ the control of ion channel homeostasis to promote viral persistence

411   (Mankouri et al., 2009). A recent study has demonstrated a relationship between ion channel

412   activity and apoptosis by showing that the caspase-dependent apoptosis induced by the

413   SARS-coronavirus 3a protein (which possesses ion channel activity) is abolished when its ion

414   channel activity is blocked (Chan et al., 2009). In the case of p7, substitution of the two

415   histidine residues (H17 and H31), which have been implicated in the gating function of p7

416   (Brohm et al., 2009; Montserret et al., 2010), with alanine, did not reduce its apoptotic

417   activity greatly (Fig. 5B), although the resultant double mutant had lost most of its ion

418   channel activity (Fig. 5A). It thus suggests that the caspase-dependent apoptosis induced by

419   p7 protein is independent of its ion channel function. Nevertheless, p7 has to be properly

420   folded and maintain the correct topology in the cell membranes in order to induce apoptosis

421   as single or double substitution of the dibasic amino acid residues R33 and R35, to glutamine

422   (Q) in the background of genotype 2a HCV p7 protein significantly reduced the level of

423   apoptosis induced by p7 (Fig. 6) and to function as an ion channel. This observation is in line

424   with a previous report that mutating the dibasic motif of p7 can substantially impact on the

425   polyprotein processing and thus distort the topology of p7 protein (Steinmann et al., 2007).

426   Recent studies (reviewed in (Eisenberg-Lerner et al., 2009; Gordy and He, 2012;

427   Zhou et al., 2011)) have shown that there seems to exist a connection between pathways that

428   regulate autophagy and apoptosis, with a number of molecules including Bcl-2 family

429   proteins, Beclin-1, caspase-8, caspase-9, phosphoinositide-3-kinase class I, and ceramide

430   implicated in the control of both apoptosis and autophagy. Apart from apoptotic or caspase-

431   dependent cell death, HCV is reported to induce non-apoptotic cell death such as autophagy

432   (reviewed in (Dreux and Chisari, 2011)). In one such report, when the autophagy machinery

433   of HCV-infected hepatocytes was interrupted, it resulted in the induction of apoptosis

434   (Shrivastava et al., 2011). Thus, it is believed that HCV exploits the autophagy pathway for

435   its propagation and survival in the host, although the biological significance of this in the

436   lifecycle HCV is still unclear (Taguwa et al., 2011). Similarly, the pro-apoptotic M2 ion

437   channel protein of influenza A virus is reported to cause accumulation of autophagosomes by

438   blocking their fusion with lysosomes which thus leads to apoptosis of the infected cells

439   (Gannage et al., 2009). Since both p7 and M2 are viroporins and are known to induce

440   caspase-dependent cell death, several experiments were therefore conducted to determine if

441   the p7 protein behaves like M2 protein of influenza A virus. Our results showed that unlike

442   M2, the overexpression of p7 did not significantly increase the conversion of LCI to LCII

443   (Fig. 8) or the accumulation of GFP-LC3 puncta (Fig. 9A) suggesting that probably p7 was

444   not involved in autophagosome maturation despite the fact that both flag-tagged M2 and p7

445   could interact with endogenous Beclin-1 in co-immunoprecipitation experiment (Fig. 9B).

446 Since a number of viral proteins including M2 have been reported to interact with Beclin-1

447 and thus interfere with the class III PI3K (UVRAG-Beclin-1-hVps34) complex's function in

448 autophagosome maturation (Dai et al., 2012; He and Levine, 2010; Munz, 2011), it is

449 therefore tempting to speculate that probably M2 and p7 bind to different domains in the

450 class III PI3K complex, with only M2 able to interact with and inhibit autophagosome

451 maturation (Gannage et al., 2010).

452 Taken together, these results indicate that HCV p7 protein induces caspase-dependent

453 apoptosis which is independent of its ion channel activity. Although p7 and M2 share some

454 functional properties in terms of ion channel and pro-apoptotic activity, they seem to differ in

455 their abilities to induce autophagic cell death. Further studies are underway in our laboratory

456 to identify residues or motifs that are responsible for the apoptosis activity of p7. The HCVcc

457 system will then be used to generate mutant virus containing mutation(s) that abolishes p7

458 pro-apoptotic activity and establish the functional significance of the apoptosis activity of p7

459 during HCV infection. In addition, identification of these residues will allow the study of the

460 interaction between HCV p7 and other viral and host factors in cell death signaling during an

461 infection.

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642 **Figure legends**

643 *Fig. 1.* Induction of apoptosis by overexpression of HCV genotype 1b (S1) and genotype 2a

644 p7 in Huh7.5 cells. (A) Apo-One fluorometric assay system from Promega Corporation

645 (Madison, WI) was used to measure the activation of caspase-3/7, which is a hallmark of

646 apoptosis, in Huh7.5 cells that were transfected with empty vector, p7 of genotype 1b (p7-1b),

647 p7 of genotype 2a (p7-2a) and Bax. All experiments were performed in triplicate, and the

648 average values with standard deviations are plotted. (B) Western blot analysis was performed

649 to determine the expression levels of the flag-tagged proteins in the different samples used in

650 (A) (top), the cleavage of endogenous PARP (middle), and the levels of endogenous actin as

651 a loading control of total cell lysates used (bottom). *P-values* indicated by asterisks are

652 considered statistically significant, *p < 0.01* (*).

653

654

655 *Fig. 2.* HCV p7 induces apoptosis via caspase-dependent pathways. (A) Apo-One

656 fluorometric assay system from Promega Corporation (Madison, WI) was used to measure

657 the activation of caspase-3/7 in Huh7.5 cells that were transfected with empty vector or flag-

658 tagged p7. The cells were treated with an irrelevant peptide, zFA-fmk (columns 1 and 2) or a

659 pan-caspase inhibitor, zVAD-fmk (columns 3 and 4). All experiments were performed in

660 triplicate, and the average values with standard deviations are plotted. (B) Western blot

661 analysis was performed to determine the expression levels of the flag-tagged proteins in the

662 different samples used in (A) (top), the cleavage of endogenous PARP (middle), and the

663 levels of endogenous actin as a loading control of total cell lysates used (bottom). Percentage

664 cleaved-PARP values are shown below the blots. *P-values* indicated by asterisks are

665 considered statistically significant, *p < 0.01* (*).

666

667   *Fig. 3.* HCV p7 induced apoptosis is via both the intrinsic and extrinsic apoptosis pathways.

668   (A) Apo-One fluorometric assay system from Promega Corporation (Madison, WI) was used

669   to measure the activation of caspase-3/7 in Huh7.5 cells that were transiently transfected with

670   empty vector or flag-tagged p7. The cells were treated with an irrelevant peptide (zFA-fmk)

671   or caspase-3, caspase-8 and caspase-9 inhibitors (zDEVD-fmk, zIETD-fmk and zLEHD-fmk).

672   All experiments were performed in triplicate, and the average values with standard deviations

673   are plotted. (B) Western blot analysis was performed to determine the expression levels of the

674   flag-tagged proteins in the different samples used in (A) (top), the cleavage of endogenous

675   PARP (middle), and the levels of endogenous actin as a loading control of total cell lysates

676   used (bottom). *P-values* indicated by asterisks are considered statistically significant, *$p <$*

677   *0.01 (*).*

678

679

680   *Fig. 4.* Detection of HCV p7 induced apoptotic cell by TUNEL assay. The DeadEnd™

681   Fluorometric TUNEL System from Promega Corporation (Madison, WI) was used to

682   measure the activation of caspase-3/7 in Huh7.5 cells that were transiently transfected with

683   empty vector or flag-tagged p7. The cells were treated with an irrelevant peptide (zFA-fmk)

684   or caspase-3, caspase-8 and caspase-9 inhibitors (zDEVD-fmk, zIETD-fmk and zLEHD-fmk).

685   At 24 h post transfection, the cells were fixed and permeabilized, and TUNEL assay was

686   performed.

687

688   *Fig. 5.* HCV p7 induced apoptosis is independent of its ion channel activity. (A) Modification

689   of membrane permeability by HCV p7 protein. 293FT cells expressing wild-type p7 protein

690   or the mutant (p7H17A31A) were treated with 0, 1, and 2 mM of hygromycin B for 30 min at

691   6 h post-transfection (lanes 1, 2, and 3). Cell lysates were prepared and the expression of the
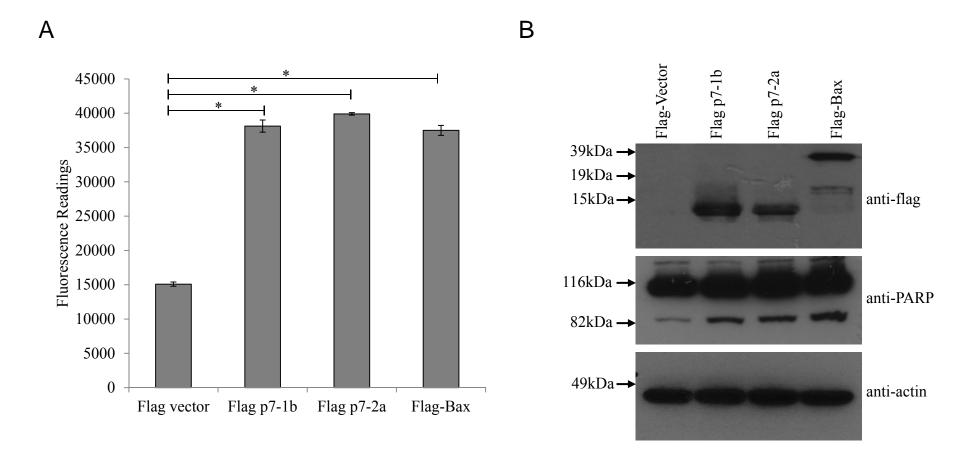
692   flag-tagged proteins was detected by immunoprecipitation with anti-Flag antibody under mild

693   washing conditions. Proteins were separated by SDS-PAGE and visualized by

694   autoradiography. The percentages of p7 and p7H17AH31A proteins detected in the presence

695   of hygromycin B were determined by densitometry and indicated at the bottom. (B) Apo-One

696   fluorometric assay system from Promega Corporation was used to measure the activation of

697   caspase-3/7 in Huh7.5 cells 24 hours post-transfection with flag-vector, wild-type p7,

698   p7H17AH31A substitution mutant and the known apoptosis inducer, Bax. (B) Western blot

699   analysis was performed to determine the expression levels of the different flag-tagged

700   proteins (top) and the cleavage of endogenous PARP, which is a substrate of activated

701   caspase-3, from 116 to 83 kDa (middle). Equal sample loading was verified by the detection

702   of endogenous actin (bottom). *P-values* indicated by asterisks are considered statistically

703   significant, *p < 0.01* (*).
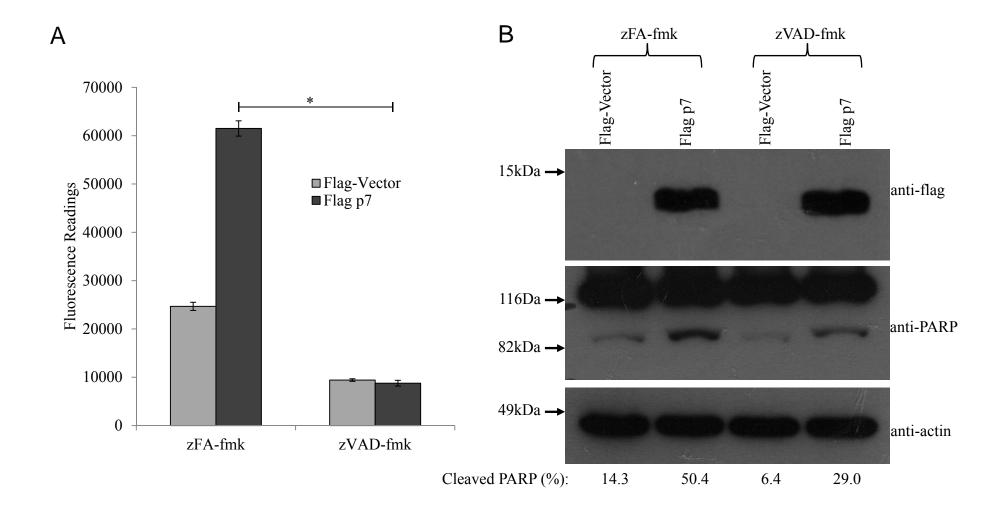
704

705

706   *Fig. 6.* HCV p7 induced apoptosis is dependent on the cellular topology of p7 protein. (A)

707   Apo-One fluorometric assay system from Promega Corporation was used to measure the

708   activation of caspase-3/7 in Huh7.5 cells 24 hours post-transfection with flag-vector, wild-

709   type p7, substitution mutants (containing either R to A or changes) and the known apoptosis

710   inducer, Bax. (B) Western blot analysis was performed to determine the expression levels of

711   the different flag-tagged proteins (top) and the cleavage of endogenous PARP, which is a

712   substrate of activated caspase-3, from 116 to 83 kDa (middle). Equal sample loading was

713   verified by the detection of endogenous actin (bottom). *P-values* indicated by asterisks are

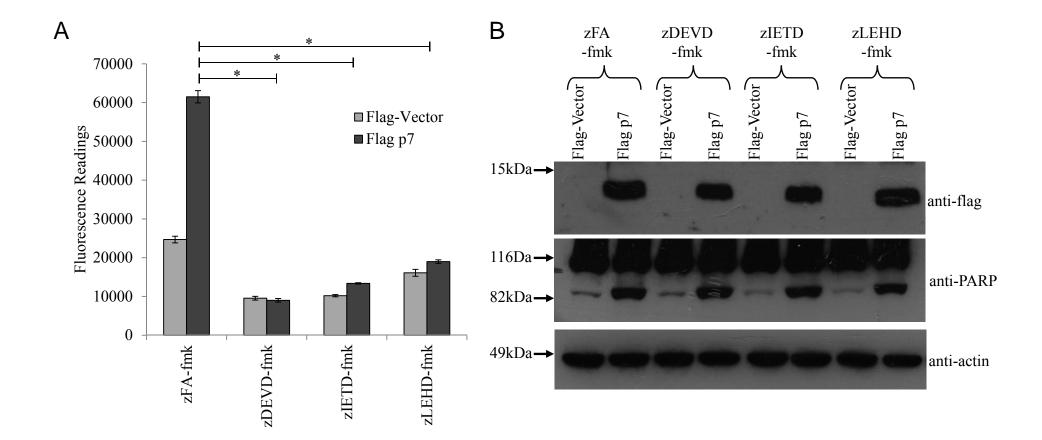714   considered statistically significant, *p < 0.01* (*).

715

716 *Fig. 7.* HCV p7 induces a higher level of apoptosis than M2 of influenza A virus. (A) Apo-

717 One fluorometric assay system from Promega Corporation was used to measure the activation

718 of caspase-3/7 in 293FT cells 24 hours post-transfection with flag-vector, flag-p7, flag-M2

719 and the known apoptosis inducer, Bax. (B) Western blot analysis was performed to

720 determine the expression levels of the different flag-tagged proteins (top) and the cleavage of

721 endogenous PARP, which is a substrate of activated caspase-3/7 (middle). Equal sample

722 loading was verified by the detection of endogenous actin (bottom). The multiple bands

723 (indicated by α) in lane 2 are oligomers of M2. *P-values* indicated by asterisks are considered

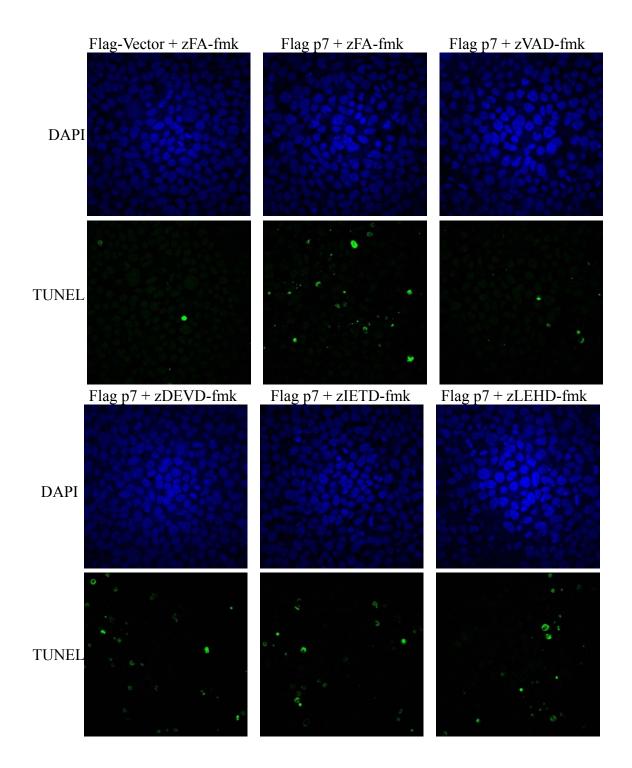724 statistically significant, *p < 0.01 (*).*

725

726

727 *Fig. 8.* HCV p7 does not induce accumulation of autophagosomes. (A) Measurement of the

728 conversion of LC3-I to LC3-II was used as an autophagic marker. 293FT cells, mock

729 transfected, or transiently transfected with flag vector, flag p7, flag-M2 and untransfected

730 cells untreated or treated with ammonium chloride were analysed by Western blot analysis

731 using anti-LC3 antibody (middle). Similarly, the expression levels of the different flag-tagged

732 proteins were determined (top). Equal sample loading was verified by the detection of

733 endogenous actin (bottom). The percentage conversion of LC3-I to LC3-II (i.e. LC3-II/LC3-

734 I+LC3-II) is calculated after densitometry was used to quantify the intensities of LC3-I and II

735 in the autoradiographs. Percentage LC3-II values are shown below the blots. (B) The mean

736 LC3-II values quantified for four independent experiments were computed after densitometry

737 and are plotted with standard deviations (error bars). *P-values* indicated by asterisks are

738 considered statistically significant, *p < 0.05 (**), p < 0.01 (*)* and no significant difference

739 (ns).

740

741   *Fig. 9.* Interaction of M2 and p7 proteins with endogenous Beclin-1 and their effects on the

742   accummulation of GFP-LC3 puncta.   (A) Immunofluorescence analysis was performed on

743   293FT cells transfected with FLAG-tagged GFP-LC3 and expression plasmids encoding

744   either FLAG-tagged M2 or p7. Twenty-four hours after transfection, cells expressing GFP-

745   LC3 (green) and p7 or M2 (red) were analyzed by fluorescence microscopy. Merged images

746   are shown below. (B) Co-immunoprecipitation of M2 and p7 proteins with endogenous

747   Beclin-1. 293FT cells were transfected with flag-vector, flag-GST (negative control), flag-

748   M2 and flag-p7. The cells were harvested at about 24 h post-transfection, lysed, and

749   subjected to IP with anti-Beclin-1 antibody and protein A agarose beads. The amount of flag-

750   tagged proteins that co-immunoprecipitated (IP) with Beclin-1 was determined by Western

751   blot analysis (WB) with an anti-flag antibody (top). The amounts of flag-tagged proteins and

752   endogenous Beclin-1 in the lysates before IP were determined by subjecting aliquots of the

753   lysates to Western blot analysis (middle and bottom). The protein marked with Greek letter

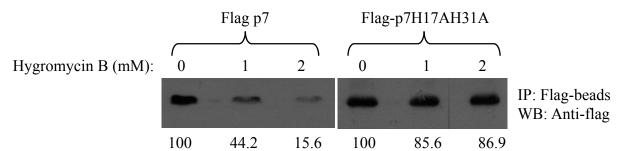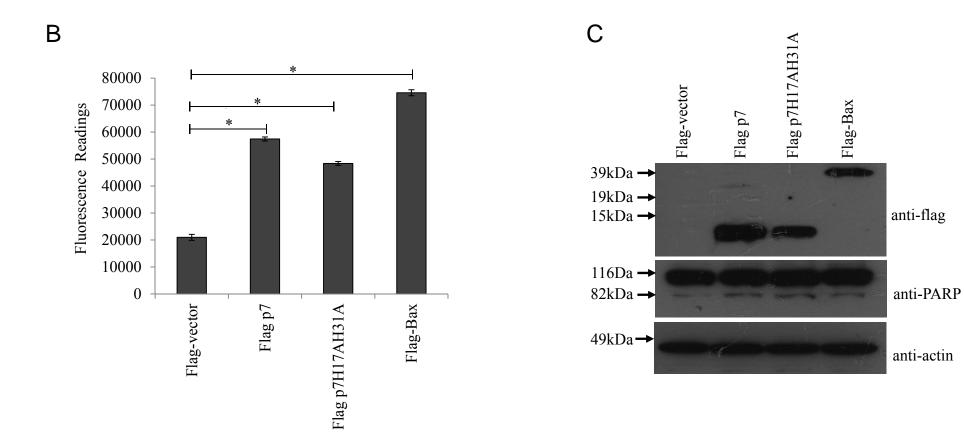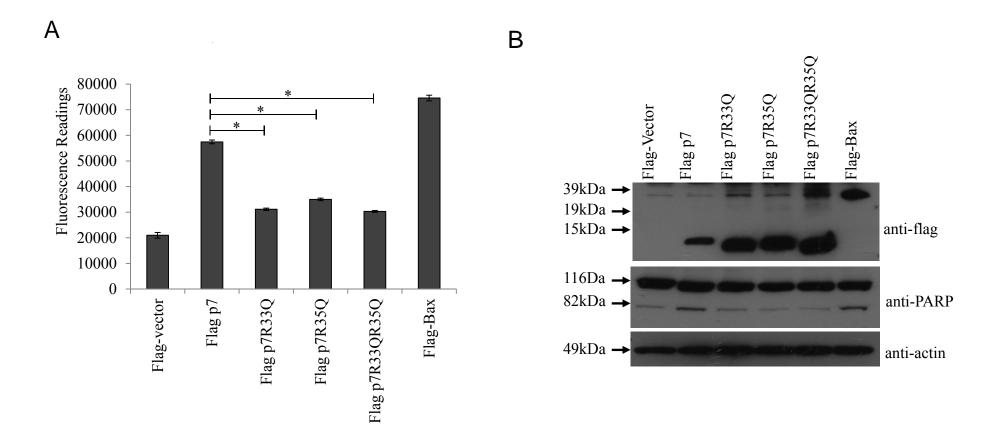754   beta (β) represents the heavy chain of the antibody used for IP.

755

756

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Flag-Vector + zFA-fmk   Flag p7 + zFA-fmk   Flag p7 + zVAD-fmk

DAPI

TUNEL

Flag p7 + zDEVD-fmk   Flag p7 + zIETD-fmk   Flag p7 + zLEHD-fmk

DAPI

TUNEL

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9