

1    **Mosquito defensins enhance Japanese encephalitis virus infection by facilitating virus**

2    **adsorption and entry within mosquito**

3    **Running title: Mosquito defensins increase JEV transmission potential**

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17     **Abstract**

18     Japanese encephalitis virus (JEV) is a viral zoonosis which can cause viral encephalitis, death

19     and disability. *Culex* is the main vector of JEV, but little is known about JEV transmission by

20     this kind of mosquito. Here, we found that mosquito defensin facilitated the adsorption of

21     JEV on target cells via both direct and indirect pathways. Mosquito defensin bound the ED III

22     domain of viral E protein and directly mediated efficient virus adsorption on the target cell

23     surface, Lipoprotein receptor-related protein 2 expressed on the cell surface is the receptor

24     affecting defensin dependent adsorption. Mosquito defensin also indirectly down-regulated

25     the expression of an antiviral protein, HSC70B. As a result, mosquitos defensin enhances JEV

26     infection in salivary gland while increasing the possibility of viral transmission by mosquito.

27     These findings demonstrate that the novel effects of mosquito defensin in JEV infection and

28     the mechanisms through which the virus exploits mosquito defensin for infection and

29     transmission.

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### 33      **Introduction**

34      Japanese encephalitis virus (JEV), a member of *Flaviviridae flavivirus*, is prevalent in  
35      Asia-Pacific tropical and subtropical regions [1-3]. JEV is mainly transmitted through  
36      mosquito bites [2, 4]. Pigs are reservoir hosts for JEV, and humans, horses and other animals  
37      are dead-end hosts [2, 5]. Because the prevention and control of JEV rely on vaccines with a  
38      limited window of protection [6-8], JEV can easily cause death or permanent disability. More  
39      than 100,000 people are at risk of JEV infection, and immunocompromised children and older  
40      individuals are at particular risk [9, 10]. The World Health Organization has reported that  
41      more than 67,900 cases of JEV infection globally each year, more than 10,000 of which are  
42      fatal. As global temperature increases, the clinical incidence of Japanese encephalitis is  
43      increasing as well, owing to an increase in the habitat range and activity of mosquitoes  
44      carrying JEV as the climate warms [2, 4, 9]. Few studies have addressed the transmission  
45      mechanism of JEV by mosquito vectors [4]. Thus, a detailed understanding of the interaction  
46      between JEV and mosquito vectors will be essential to improve control of JEV transmission.  
47      *Culex* is the principal vector of JEV [11, 12]. The virus can spread throughout the  
48      mosquito body, including salivary glands [13]. When an infected mosquito bites humans or

49 animals, the virus is transmitted to the skin through the saliva. The mosquito vector also  
50 induces an immune response to JEV [14-16]. For example, C-type lectin and a series of  
51 proteins increase rapidly after infection [17, 18]. C-type lectin plays an important role in  
52 infection by JEV and other *Flaviviridae* viruses in mosquitoes, but the role of defensin has  
53 not yet been clearly characterized.

54 Defensins are antimicrobial peptides consisting of 25–60 amino acids that are produced  
55 by innate immune system [15, 19]. Defensin is one of the crucial immune effectors in insects  
56 [20]. The antiviral effects of defensins have been well described in mammalian cells. Human  
57 defensins have been reported to inhibit herpes simplex virus type 2 (retrocyclin-1,  
58 retrocyclin-2) [21], human immunodeficiency virus (human beta defensin-1, human beta  
59 defensin-2, Human beta defensin-3) [22, 23] and other viruses. However, human beta  
60 defensin-6, expressed by adenovirus vectors, enhances parainfluenza virus type 3 replication  
61 [24]. Normally, mammalian defensins can directly destroy the virus particles by binding to  
62 the surface of envelope protein. They can also interact to the cell surface receptor and  
63 influence cell signal transduction [19, 25]. Although there are many differences between the  
64 mammalian and mosquito immune systems, defensins are considered important effectors in

65 the mosquito immune response. Therefore, the role of mosquito defensins during the process

66 of JEV infection requires further study.

67 In this study, we observed complex roles of mosquito defensin in JEV infection: a weak

68 antiviral effect and a strong effect enhancing binding. In the latter, defensin directly binds the

69 ED III domain of the viral E protein and promotes the adsorption of JEV to target cells by

70 interacting with lipoprotein receptor-related protein 2 (LRP2), thus accelerating virus entry.

71 Mosquito defensin also down-regulates the expression of the antiviral protein HSC70B on the

72 cell surface, thus facilitating virus adsorption. Together, our results indicate that the

73 facilitation effect of mosquito defensin plays an important role in JEV infection and potential

74 transmission.

75

## 76 **Results**

### 77 **JEV infection up-regulates defensin expression *in vivo* and *in vitro***

78 Defensin is one of the major innate immunity effectors in mosquitoes. To study the role

79 of defensin in JEV infection, we first assessed the expression of defensin in *Culex pipiens*

80 *pallens* (*Cpp*) which is the natural vector of JEV after infection. Five-day-old female

81 mosquitoes after emergence were infected by a microinjector with a dose of 1000 MID<sub>50</sub> [18].

82 The mosquitoes were collected 4, 7 and 10 days after injection, and the JEV E mRNA levels

83 in the whole body, salivary gland and midgut were determined. JEV E mRNA showed higher

84 levels in the salivary gland than in the whole body and midgut (Fig. 1A). At 10 days, the JEV

85 E mRNA level increased dramatically, thus indicating that the virus reproduced rapidly

86 during this period. For instance, JEV E mRNA levels increased by 9.7- and 11.7-fold at 10

87 days compared with 7 days in the whole body and midgut, respectively. A greater increase

88 was observed in the salivary gland, reaching 14.9 fold at 10 days compared with 7 days.

89 Because high virus levels in mosquitoes were observed 7 and 10 days post JEV infection, we

90 then determined the defensin A and total defensins mRNA level in the whole body on 7 and

91 10 days. *Cpp* defensin A mRNA levels on both days were significantly higher in the JEV

92 infection group than the control group, although the level decreased slightly at 10 days (Fig.

93 1B). Change levels of total defensins showed the similar trend (Fig. 1B). Furthermore, we

94 compared *cpp* defensin A and total defensins mRNA levels in the whole body, salivary gland

95 and midgut. Defensin A and total defensins mRNA showed similar levels of up-regulation in

96 the salivary gland and whole body, which were higher than those in the midgut (Fig. 1C).

97 Significantly higher mRNA levels ( $p<0.005$ ) were observed in the JEV infection group than  
98 the control group for whole body, salivary and midgut. This suggested that *CpP* defensin A  
99 expression was positive correlated with JEV infection in mosquitoes.

100 The gene encoding *cpp* defensin was not found in the NCBI database. According to

101 PCR amplification (Fig. S1A), sequencing and BLASTn (<https://blast.ncbi.nlm.nih.gov>)

102 results, we found two gene types of defensin: defensin A (submitted to NCBI with accession

103 number MH756645) and an incomplete information defensin. The mature protein regions of

104 these two defensins shared 99.5% sequence similarity (Fig. S1B). We designed specific

105 primers (Table. S1, Fig. S2) for real-time PCR detection according to the *CpP* defensin A

106 sequence. However, no specific primers were available for the unnamed defensin, because

107 scarce specific sequence was obtained. Therefore, we quantified the mRNA copy number of

108 total and type A defensins to determine which subtype is the primary defensin in *cpp* (Fig.

109 S1C). The fold change of mRNA levels of defensin A were significantly higher (7 days,

110  $p<0.05$ ) than or similar (10 days) to the total defensin levels (Fig. 1B), thus it implied that

111 defensin A accounts for the majority of total defensins. To analyze whether defensin

112 functioned universally among organisms, we aligned the defensin protein sequences of

113 mosquito vectors of flavivirus. We also quantified the mRNA copy number of total and type  
114 C defensins to determine which subtype is the primary defensin in *Aedes albopictus* (*aa*) (Fig.  
115 **S1C**). *Aedes* defensin C is the major type of defensin in C6/36 cell, a cell line from *Aedes*  
116 *albopictus*. The sequence similarities were all above 97.6% between mosquito vectors (Fig.  
117 **S1D**), suggesting that mosquito defensins serve similar functions. In contrast, the sequence  
118 similarities were significantly low between mosquitoes and human (Fig. **S1E**). We then used  
119 *cpp* defensin A (accession number MH756645) and *aa* defensin C (accession number  
120 XP\_019527114.1) to study the functions of defensin in JEV infection within mosquito  
121 vectors.

122 To confirm the up-regulation of defensin in different mosquito vectors caused by JEV  
123 infection, we infected C6/36 cells with JEV *in vitro*. JEV E mRNA levels increased from 24 h  
124 to 120 h post JEV infection (Fig. **1D**). We further analyzed the change of total defensins and  
125 primary defensin (*aa* defensin C, Fig. **S1C**) after JEV infection. *aa* defensin C mRNA levels  
126 were up-regulated to 2.75-, 11.9-, 19.7-fold in JEV infection compared with mock infection at  
127 1, 3 and 5 days, respectively (Fig. **1E**). Also total defensins were up-regulated after JEV  
128 infection (Fig. **1E**). Together, our results indicated that defensin levels were up-regulated after

129 both *in vivo* and *in vitro* infections.

130

131 **Mosquito defensin shows species specificity in facilitating JEV infection**

132 Mature defensin is an extracellular protein which length is less than 60 amino acids. To

133 confirm the function of defensin on JEV infection, we synthesized mature defensin peptides

134 with high purity ( $\geq 99\%$ ) to perform further analysis. Scrambled defensin peptides were used

135 as controls. *Cp* defensin A and *aa* defensin C peptides were used in both *in vivo* and *in vitro*

136 experiment. Defensins and JEV were pre-mixed before injection into mosquitoes.

137 Unexpectedly, in *in vivo* experiment, JEV E mRNA levels increased by 2.95- and 6.13-fold in

138 the *cpp* defensin A treated groups compared with the control groups at 7 and 10 days post

139 infection, respectively (Fig. 2A). And the same changing trend of JEV level was observed in

140 *aa* defensin C treated group (Fig. 2A). We also confirmed this enhancement of defensins on

141 JEV infection by RNA interference. siRNA sequences target *Cp* defensins or *Cp* defensins

142 A were designed and used in *in vivo* RNA interference. JEV E mRNA levels were decreased

143 by more than 5 fold in *Cp* defensin siRNA groups and more than 3 fold in *Cp* defensin A

144 siRNA groups compared to scramble group (Fig. 2B, Fig. S3A). Indirect immunofluorescence

145 assay (IFA) analysis also showed higher JEV E levels in the mosquito defensin treated cells  
146 than the control (Fig. 2C), and lower JEV E levels in the mosquito defensin knockdowned  
147 cells than the control (Fig. 2C).

148 To compare the role of defensin from different species, human defensin  $\beta$ 2 showed high  
149 antiviral activity was synthesized [25, 26]. Firstly, we compared the effects of *aa* defensin C,  
150 *Cpp* defensin A and human defensin  $\beta$ 2 on C6/36 cells. *aa* defensin C enhanced JEV infection

151 on C6/36, as indicated by both JEV E mRNA (4.88 fold) and TCID50 (1.3 titer) levels (Fig.  
152 2D i and ii). Treating with *Cpp* defensing A also resulted in the enhancement of JEV

153 infection. In contrast, human defensin  $\beta$ 2 inhibited JEV replication on C6/36 cell, thus  
154 demonstrating that defensins from different species have diverse functions in JEV infection

155 (Fig. 2D i and ii). To confirm this effect of mosquito defensin, we used siRNAs target  
156 defensin of C6/36. JEV was inoculated and detected after siRNAs transfection. JEV E mRNA

157 levels decreased by 4.7 to 6 folds in *aa* defensin interference groups, and decreased by 2.3 to  
158 3.1 folds in *aa* defensin C interference groups respectively (Fig. 2E i and ii, Fig. S3B). These

159 results were consistent with the *in vivo* data.

160 To obtain detailed insight into the function of mosquito defensin, we analyzed the effects

161 of mosquito defensin on mammalian cells contain Vero and BHK-21. *aa* defensin C reduced  
162 the JEV replication by 2.2 to 2.7 folds (Fig. 2F i and Fig. 2G i) and decreased JEV TCID<sub>50</sub>  
163 levels by 0.6 to 0.8 titers (Fig. 2F ii and Fig. 2G ii), thus indicating that it inhibits JEV  
164 infection in mammalian cells as human defensin dose. Although the inhibition ability of *aa*  
165 defensin C was lower than that of human defensin  $\beta$ 2, it still inhibited JEV replication.  
166 Therefore, the facilitation effects of mosquito defensin on JEV were valid only on mosquitoes  
167 and mosquito cells. To confirm that the effect of defensins was not due to cytotoxicity, we  
168 measured the IC<sub>50</sub> of each defensin through MTT assays. The results showed that defensins  
169 had no significant cytotoxicity on cells (Table. S2).

170

### 171 **Mosquito defensin enhances JEV adsorption to target cells**

172 To study the exact mechanisms of mosquito defensin facilitates JEV infection, we  
173 analyzed the influence of *aa* defensin C on different infection steps on C6/36 cell. As  
174 infection steps can be measured by temperature and time shift, we detected adsorption,  
175 uncoating and replication of JEV [27]. Adsorption was determined to be a key step in the  
176 facilitation effect (Fig 3A). Next, we detected JEV adsorption at different time points. JEV

177 mixed with defensin or scrambled peptides was inoculated to C6/36 cells for different time  
178 points at 0°C. After being washed with PBS for three times, cells with absorbed JEV were  
179 collected. JEV E mRNA levels were determined by real-time PCR. C6/36 cells treated with  
180 *aa* defensin C showed significantly higher JEV E mRNA levels at 4 and 6 h post adsorption,  
181 proof that defensin enhanced JEV adsorption to C6/36 (Fig. 3B). IFA showed that JEV  
182 adsorption greatly increased in a time course of *aa* defensin C treatment (Fig. 3C and Fig.  
183 3D). Both nuclear staining (DAPI) and membrane staining (Did) of C6/36 cell were  
184 conducted in IFA absorption analysis. There was stronger JEV adsorption in the *aa* defensin  
185 C groups than the control groups at each time points in both DAPI staining (Fig. 3C) and Did  
186 staining (Fig. 3D) cells. To study how mosquito defensin facilitated JEV adsorption, FITC  
187 labeled *aa* defensin C was used. Defensin-FITC and JEV were mixed before incubation at  
188 0°C. After incubation, unabsorbed defensin and JEV were washed by PBS for five times. The  
189 cells were collected at the indicated time points to observe the co-localization of defensin and  
190 JEV. Strong co-localization on the cell surface was observed between *aa* defensin C and JEV  
191 (Fig. 3E) and increased over time. Thus, the facilitation effect of mosquito defensin on JEV  
192 was attributed to the binding between them. Additionally, JEV mixed with *cpp* defensin A

193 showed high adsorption capacity in salivary glands ([Fig. 3F](#)). Take together, our results

194 indicated that mosquito defensin is able to bind JEV and facilitate virus adsorption.

195 The interaction between defensin and JEV was also confirmed by ELISA. The plate

196 wells were coated with *aa* defensin C, incubated with JEV and next incubated with anti-JEV

197 antibody. As expected, JEV bound defensin efficiently. Even with a 250 ng defensin coating

198 treatment, the JEV level was significantly higher than that in the control group ([Fig. 3G](#)). To

199 determine the adsorption capacity of the JEV-defensin complex to C6/36 cells, we coated

200 plate wells with fresh C6/36 cells after polylysine treatment, added pre-mixed defensin and

201 JEV, and detected JEV with anti-JEV monoclonal antibody. In accordance with the results of

202 qPCR and IFA, the interaction of defensin with JEV significantly enhanced JEV adsorption to

203 C6/36 cells ([Fig. 3H](#)).

204 Based on the previous results, we deduced that mosquito defensin can efficiently bind to

205 cell surface. The interaction of mosquito defensin with the cell surface was assessed through

206 ELISA. Defensin directly interacted with C6/36, and a higher FITC value than that of the

207 control was observed ([Fig. 3I](#)). This finding implied that the facilitation effect of defensin on

208 JEV was caused by increasing the affinity of JEV on the cell surface.

209

210 **Defensin directly binds the JEV ED III domain**

211 Defensins can bind to viral envelope protein [25]. To precisely understand the interaction

212 mechanisms of JEV enhancement mediated by defensin, we expressed the three structural

213 proteins (C, prM and E) of JEV and the exposure area (ED III) of E protein through an S2

214 insect protein expression system [28, 29], and further purified these proteins via 6×His

215 agarose. To analyze the interaction between viral proteins and defensins, two ELISA methods

216 were used. The plate wells were coated with defensin, incubated with purified proteins and

217 detected by corresponding antibodies. Scrambled defensin was used as control. Absorbance

218 results showed high affinity between *aa* defensin C and E protein or ED III protein, which

219 were ~0.95 and ~1.17, respectively (Fig. 4A). Consistent results were observed in

220 defensin-FITC testing. The plate wells were coated with purified viral proteins and then

221 incubated with *aa* defensin C-FITC or scrambled defensin-FITC. E and ED III proteins

222 showed higher fluorescence values of 332 and 369, respectively (Fig. 4B). Both tests

223 suggested that the ED III domain of E protein is the key region involved in *aa* defensin C and

224 JEV binding. Subsequently, purified E and ED III proteins were mixed with *aa* defensin C

225 and used to inoculate C6/36 cells at 0°C for 4 h. Unabsorbed defensin and JEV were removed  
226 by washing with PBS after incubation. The effect of *aa* defensin C on facilitating E and ED  
227 III adsorption was observed by fluorescence microscope ([Fig. 4C](#) and [Fig. 4D](#)). E proteins  
228 and ED III proteins bound more efficiently to the C6/36 cell with *aa* defensin. Additionally,  
229 *aa* defensin C showed co-localization with E or ED III protein on the C6/36 ([Fig. 4C, merge](#)  
230 [panel](#)). The same results were observed in membrane stained C6/36 cells. E proteins and ED  
231 III proteins bound more efficiently to the C6/36 cell surface with *aa* defensin, and *aa* defensin  
232 C also showed co-localization with E or ED III protein on the C6/36 surface ([Fig. 4D](#)). Thus  
233 indicating that the ED III domain of the JEV E protein responsible for binding with *aa*  
234 defensin C.

235

### 236 **LRP2 is responsible for mosquito defensin mediated JEV adsorption**

237 As an extracellular protein, defensin has been reported to interact with receptors on the  
238 cell surface and consequently affect intracellular signaling networks. To define the  
239 relationship of defensin/cell surface receptor and adsorption enhancement, we analyzed  
240 cell-surface receptors that interact with defensin. We knockdowned the expression of a series of

241 potential receptors on the cell surface through RNA interference (RNAi) and found that

242 lipoprotein receptor-related protein 2 (LRP2) responsible for defensin binding [30, 31]. The

243 results indicated that LRP2 interfered with the interaction between defensin-FITC and C6/36 cells

244 (Fig. 5A and Fig. 5B), thus indicating that LRP2 related to the adsorption of extracellular

245 defensin. We further studied the role of LRP2 on JEV adsorption mediated by defensin. Based on

246 significantly RNA knock down (Fig. S3C), No differences were observed between cells with or

247 without LRP2 interference when infected with JEV alone (Fig. 5C). However, when C6/36 cells

248 were incubated with mixed defensin and JEV, a lower JEV level was observed in LRP2 interfered

249 cells. The JEV mRNA level on LRP2 interference cells was 2.8 fold lower than that of the

250 scramble (Negative control, NC) interference group (Fig. 5C), and both the TCID<sub>50</sub> level and

251 fluorescence value decreased significantly (Fig. 5D and Fig. 5E). In *in vivo* mosquito experiments,

252 the mosquitoes were inoculated with LRP2 or NC siRNA for 3 days (Fig. S3D), and inoculated

253 with defensin and JEV mixture. Whole body samples were collected at 3 days after infection. The

254 JEV mRNA level was significantly lower in LRP2-interference group than in the NC group (Fig.

255 5F), thus indicating that LRP2 participated in defensin mediated viral adsorption. Additionally, the

256 results of indirect immunofluorescence were in accordance with the above-mentioned results.

257 Take together, our findings indicated that LRP2 is the cell surface factor responsible for defensin

258 mediated JEV adsorption. LRP2/defensin is a pathway mediates JEV adsorption in mosquito.

259 Lipoprotein receptor-related protein 4 (LRP4) and CXCR4 also showed binding activity with

260 defensin, but this activity did not influence JEV adsorption (Data not shown).

261

262 **Mosquito defensin down-regulates the expression of HSC70B on the C6/36 surface and**

263 **reduces antiviral activity of cell**

264 Defensin can interact with cell-surface receptors and consequently affect signal

265 transduction in cells. Therefore, we employed stable isotope labeling with amino acids in cell

266 culture (SILAC) labeling and mass spectroscopy (MS) methods ([Fig. 6A](#)) to determine

267 whether mosquito defensin influences the expression of proteins on cell surface and

268 consequently affects JEV adsorption [32]. Briefly, C6/36 cells were continuously passaged on

269 media with light, medium and heavy stable isotopes. After more than 99.0% cells were

270 labeled with stable isotopes, the cells were then grouped, inoculated with JEV or defensin,

271 and collected according to the plan ([Fig. 6A](#)). Cell membrane proteins were extracted for MS

272 analysis. The results showed that HSC70B, a potential mosquito antiviral protein [33], was

273 significantly down-regulated in all defensin, JEV, and defensin + JEV treatments (Fig. 6B).

274 We prepared a rabbit polyclonal antibody against C6/36 HSC70B (UniProt accession number

275 A0A0E3J979) according to the MS results (Fig. S2). Western-blot analysis validated the

276 down-regulation of HSC70B on the C6/36 cell surface in all three treatments (Fig. 6C).

277 Because HSC70B is a potential mosquito antiviral protein, we tested the function of

278 mosquito HSC70B on JEV adsorption and infection. siRNA targeting C6/36 HSC70B was

279 transfected, and the interference efficiency of HSC70B on C6/36 was detected through

280 real-time PCR analysis (Fig. 6D). Afterward, JEV was inoculated to HSC70B interfered

281 C6/36 cells. The JEV adsorption capacity in HSC70B-interference cells was significantly

282 higher than that in NC-interference cells ( $p<0.05$ ) (Fig. 6E). Additionally, JEV replication

283 level was detected in C6/36 cells treated with HSC70B siRNAs and defensin peptides.

284 Compared to NC group, HSC70B interference significantly heightened the enhancement

285 function of defensin (Fig. 6E). Similarly, we designed siRNA targeting the homologous gene

286 of *cpp* HSC70B. siRNA was injected into *cpp*, and the interference efficiency of HSC70B

287 was detected through real-time PCR (Fig. 6F). JEV mRNA levels were detected at 6 days

288 post infection. Likewise, HSC70B-interfered mosquitoes produced more JEV copies than NC

289 group (Fig. 6G).

290 Two mechanisms underlying the facilitation effect were identified. One was a direct

291 binding effect, enhancing JEV affinity to the cell surface. The other was an indirect effect,

292 weakening the host defense by down-regulating antiviral HSC70B expression.

293

294 **Mosquito defensins facilitate JEV dissemination in salivary gland**

295 To assess the transmission potential of JEV enhanced by mosquito defensins, we

296 detected the virus levels within salivary gland of defensin-treated mosquitoes [34, 35]. Both

297 microinjection and blood meal methods were used in this experiment. JEV and mosquito

298 defensin were mixed before inoculation. Mosquitoes injected with JEV and defensin peptide

299 were collected at 7 or 10 days post infection. Fresh salivary glands were isolated and detected

300 by using real time PCR. JEV level were significantly increased in *Cpp* and *aa* defensin groups

301 (Fig. 7A). JEV level in *Cpp* defensin group indicated 3.5 fold higher at day 7 post infection

302 and 3.1 fold higher at day 10 post infection than that of scramble defensin group in salivary

303 gland. *aa* defensin showed the same role as *Cpp* defensin did. We further employed blood

304 meal to measure the effect of defensin in JEV dissemination in mosquito salivary gland. Five

305 day-old female Mosquitoes were deprived of sucrose and water 24 h prior to blood meal.

306 Mosquitoes were then feed with infectious blood with JEV and defensin peptides for 2 h.

307 Fresh blood was collected from health mice and delivered through Hemotek membrane

308 feeding apparatus. 2ml blood with JEV ( $5 \times 10^6$  TCID<sub>50</sub>) and peptide (200  $\mu$ M) were used for

309 each groups. JEV level in *Cp* defensin group showed 4.2 fold higher at day 7 and 10 post

310 infection than that of scramble defensin group in salivary gland (Fig. 7B). JEV level in *aa*

311 defensin group also showed higher results than scramble group. These results implied that

312 mosquito defensins facilitate JEV dissemination in mosquitoes and increase transmission

313 potential after infection.

314

## 315 **Discussion**

316 JEV is a serious mosquito borne disease common in Asia-Pacific tropical and subtropical

317 regions [2, 9, 10]. More than 100,000 people are at risk. Moreover, JEV can cause death or

318 permanent sequelae. Pigs are the reservoir host of the virus. Humans, horses and other

319 animals are dead-end hosts. Mosquitoes, especially *culex*, are the most important vector [4].

320 At present, the prevention and control of JEV mainly relies on vaccine immunization, whose

321 protection time is limited. JEV remains a threat to health and even life for  
322 immunocompromised children or older people [6, 7]. With the increasing problem of global  
323 warming, the clinical incidence of JEV is increasing [36]. Few mechanistic studies have  
324 focused on the JEV transmission by mosquito vectors. It is of practical significance to  
325 understand the interaction between JEV and mosquito vectors and the immune escape mode  
326 of JEV in controlling this mosquito borne disease.

327 In this study, we analyzed the gene expression of defensin from *Cpp* and *aa*. Defensin A  
328 and an unnamed defensin from *Cpp*, defensin A, B and C from *aa* shared high sequence  
329 similarity, thus indicating similar functions of these defensins. Subsequently, we confirmed  
330 that defensin A and defensin C are the main defensin types of *cpp* and *aa*, respectively. Given  
331 the high similarity of the amino acid sequences, we synthesized only *cpp* defensin A and *aa*  
332 defensin C in further studies. The nucleotide sequence of *cpp* defensin A (number  
333 MH756645) has been submitted to the NCBI database.

334 The up-regulation of defensin after JEV infection was consistent with reports on other  
335 flavivirus viruses [37]. The highest up-regulation was observed at 7 days post infection. From  
336 the organism perspective, the defensin in the salivary gland and whole body was up-regulated

337 more than that in the midgut. JEV replication in salivary gland, the most sensitive tissue to

338 JEV [18], was positively correlated with defensin level.

339 The mature peptide of defensin was utilized to study the role of mosquito defensin in

340 JEV infection [25, 38]. In general, defensin is fewer than 60 amino acids and is processed

341 from a precursor protein. In this study, mosquito defensin and human defensin  $\beta$ 2 were

342 composed of 40 amino acids and 34 amino acids [39], respectively. Only 11% sequence

343 similarity was identified between mosquito and human defensin. Unexpectedly, mosquito

344 defensin facilitated JEV infection, in contrast to human defensin, but the facilitation effect

345 was exerted only on mosquito cells or mosquitoes. Thus, JEV utilized the host defense

346 system, reflecting its “intelligence” in infection [40]. However, mosquito defensin inhibited

347 JEV infection in mammalian cells, thus indicating its varied mechanisms of action and the

348 complicated interaction between virus and host [19].

349 Further analysis demonstrated that mosquito defensin facilitated JEV adsorption to target

350 cells by directly binding JEV virions [27, 41]. By screening JEV structural proteins, we found

351 that mosquito defensin bound the ED III domain of JEV E. ED III is a crucial domain of JEV

352 that is responsible for the production of neutralizing antibody [42]. The antiviral effect of

353 mosquito defensin on JEV is likely to be due to its binding the ED III domain and subsequent  
354 virion destruction [19, 25]. Because mosquito defensin facilitated JEV infection, the binding  
355 of defensin and ED III can be inferred to have only weak antiviral effects. Nevertheless, this  
356 binding enhanced virion adsorption ability to a large extent. The broad transmission of JEV  
357 by mosquitoes is ascribed to both the crude immune system of mosquitoes and the infection  
358 strategy of the virus. Mosquito defensin could improve the adsorption ability of JEV on target  
359 cells. Additionally, ELISA results showed that high concentration of mosquito defensin  
360 interacted with the target cells without the assistance of viruses.

361 Defensin receptors expressed on the cell surface may lead to enhanced adsorption. We  
362 scanned the potential cell-surface receptor proteins of defensin through RNAi and found that  
363 the LRP2-defensin pathway was responsible for JEV adsorption. In mammalian animals,  
364 LRP2 is the receptor for defensin, regulating the contraction of smooth muscle cells by  
365 combining with human alpha defensin [30, 31]. However, the roles of LRP2 in mosquitoes  
366 have not been reported. In the present study, we demonstrated that LRP2 participates in JEV  
367 adsorption mediated by defensin. JEV first binds defensin, and then, owing to the affinity of  
368 defensin for LRP2, the defensin/JEV complex adsorbs to the cell surface more readily,

369 thereby increasing the chance of infection. This proposed mechanism of promotion of JEV  
370 infection by defensin/LRP2 is similar to that for JEV and WNV mediated by C type  
371 lectin/PTP-1. That is, virus first combines with extracellular secrete proteins with high  
372 affinity to cells, and this is followed by binding to cell surface receptor to infect target cells.

373 Given that mosquito defensin directly interacts with mosquito cell surface receptors, we  
374 analyzed how it regulates cell surface proteins. The changes in cell surface proteins were  
375 determined through SILAC and MS analysis [32]. We identified a potential antiviral protein,  
376 HSC70B, that is significantly down-regulated by defensin or JEV treatment [33]. HSC70B  
377 inhibited JEV adsorption, as demonstrated through an RNAi approach, thus indicating that  
378 mosquito defensin indirectly affects JEV adsorption by regulating cell surface antiviral  
379 protein expression. However, this indirect effect was found to be lower than the direct  
380 defensin binding effect. Together, our findings indicated that the effect of mosquito defensin  
381 on JEV is composed of weak antiviral effect, direct binding enhancement and indirect  
382 immune regulation. Curiously, both defensin and HSC70B are antiviral proteins in mosquito,  
383 but it looks like they could't work together on JEV infection. We did not identify the  
384 mechanisms through which defensin down-regulates HSC70B, because of the limited

385 information available on the relevant signal pathways. We deduced that there is a negative

386 feedback mechanism between HSC70B and defensin [43], thus implying that an increase in

387 defensin would decrease HSC70B level. Another possibility may be that HSC70B has

388 varying functions in different conditions, except for the antiviral effect.

389 JEV infection up-regulated mosquito defensin expression in the salivary gland and

390 defensin also heightened the JEV dissemination in salivary gland, thus suggesting that the

391 defensin may be influence the transmission of JEV by mosquito. Further research on

392 mosquito defensin in JEV cross-species transmission is needed.

393 To our knowledge, this is the first report on the effects of mosquito defensin on JEV

394 infection in mosquito vectors, revealing a new immune escape mechanism of JEV infection

395 and transmission. This study broadens our knowledge of transmission of JEV as well as other

396 mosquito borne viruses, providing novel insights into viral transmission mechanisms.

397

398 **Materials and methods**

399 **Ethics statement**

400 All animal experiments were performed in compliance with the Guidelines on the

401 Humane Treatment of Laboratory Animals (Ministry of Science and Technology of the  
402 People's Republic of China, Policy No. 2006 398) and were approved by the Institutional  
403 Animal Care and Use Committee at the Shanghai Veterinary Research Institute (IACUC No:  
404 Shvri-Pi-0124).

405

406 **Cells, defensin and viruses**

407 Baby hamster kidney (BHK-21) and African green monkey kidney (Vero) cells were  
408 purchased from the ATCC (Rockville, Maryland) and maintained in Dulbecco's modified  
409 Eagle's medium (DMEM) supplemented with 10% FBS at 37°C in a 5% CO<sub>2</sub> incubator.  
410 C6/36 cells (ATCC) were cultured in RPMI-1640 medium supplemented with 10% FBS at  
411 28°C.

412 Mature *Cp* defensin A (NCBI accession number: MH756645), *aa* defensin C (NCBI  
413 accession number: XP\_019527114.1), human defensin β2 (NCBI accession number:  
414 NP\_004933.1) and scrambled defensin peptides (purity ≥ 99%) were synthesized by  
415 WC-Gene Biotech Ltd. (Shanghai, China). The amino acid sequences are shown in Table S3.  
416 The defensins were dissolved in DMSO (for cell, *ex vivo* or *in vivo* experiments) or PBS (for  
417 ELISA detection) and stored at room temperature. Defensins labeled with FITC were kept in

418 the dark at room temperature.

419 JEV strain N28 (NCBI accession number: GU253951.1) was stored in our laboratory and

420 propagated in C6/36 cells. The TCID<sub>50</sub> and MID<sub>50</sub> of the virus were measured in BHK-21

421 cells or female mosquitoes and calculated by the Reed-Muench method [17, 44].

422

423 **Infection and RNA interference *in vitro***

424 Defensins or scrambled defensin peptides were pre-mixed with JEV (MOI=0.1) at 4°C,

425 then inoculated into cells. C6/36 cells were incubated at 28°C for 2 h. Vero and BHK-21 cells

426 were incubated at 37°C for 2 h. At 24–120 h post infection, the supernatant or cells were

427 collected. Viral titer was determined by TCID<sub>50</sub> method and mRNA expression levels were

428 measured by real-time PCR. To determine JEV adsorption, defensins were pre-mixed with

429 JEV at 4°C for 2 h. C6/36 cells were incubated with the mixture on ice for different times.

430 Unabsorbed JEV was removed by washing with PBS for three times. The cells were collected

431 for JEV E mRNA quantification or other measurements.

432 For the *in vitro* RNA interference, siRNA ([Table S1](#)) was transfected into C6/36 cells

433 with Cellfectin II reagent (Invitrogen). JEV was inoculated at 24 h post transfection. At 72 h

434 post infection, the cells were collected. Total RNA was isolated, and the viral or gene load

435 was determined by real-time PCR.

436

437 **Infection and RNA interference *in vivo***

438 For *in vivo* experiments, 10-fold serial dilutions were made from a  $10^{9.3}$  TCID<sub>50</sub> JEV

439 stock. Cold-anesthetized 5 day old female mosquitoes were randomly divided into various

440 groups ( $n \geq 13$ ). Both microinjection and blood meal methods were carried out in infection

441 experiment. For microinjection, the mosquitoes were infected by microinjection (250 nL) into

442 the thorax. An Eppendorf CellTram oil microinjector and 15  $\mu$ m needles were used for

443 injecting the mosquitoes. Control mosquitoes were injected with an equivalent volume of

444 PBS [17, 18, 45]. The mosquitoes were harvested, and the viral loading was quantified. For

445 blood meal, fresh blood of specific pathogen free mouse was collected in tubes with

446 anticoagulant. Virus or defensin peptides were mixed and added into fresh blood before

447 feeding. 2 ml blood was used in blood meal by Hemotek FU1 Feeder for each group [34, 46].

448 *In vivo* RNAi was performed as described previously [18]. The siRNA targeting the *Cpp*

449 genes was synthesized by Genepharma (Shanghai, China). The sequences are shown in Table

450 **S1.** For RNAi and virus challenge, female mosquitoes at 5 days after eclosion were injected  
451 into the thorax with 2  $\mu$ g dsRNA in 250 nL PBS. After a 3 day recovery period, the  
452 mosquitoes were microinjected with JEV at different MID<sub>50</sub> in 250 nL PBS for functional  
453 studies.

454

455 **RNA isolation and real-time PCR**

456 For real-time PCR, RNA was extracted from cell suspensions or mosquito samples by

457 Qiagen total RNA isolation kit according to the manufacturer's instructions. The RNA

458 concentration was measured by NanoDrop spectrophotometer. cDNA was generated by RT

459 Master reverse transcription kit (Takara) according to the manufacturer's instructions.

460 Real-time quantitative PCR experiments were performed in ABI Prism 7500

461 sequence-detection system (Applied Biosystems, Foster City, CA) with SYBR Green PCR

462 Master Mix (Takara) according to the manufacturer's instructions. The primer sequences are

463 listed in Table 1. The thermal cycling conditions were as follows: 10 min at 95°C, followed

464 by 40 cycles of 95°C for 5 s and 60°C for 1 min. All experiments were performed in

465 triplicate, and gene expression levels are presented relative to those of  $\beta$ -actin. The fold

466 change in relative gene expression compared with the control was determined with the

467 standard  $2^{-\Delta\Delta C_t}$  method.

468

469 **Virus titer**

470 Supernatants were harvested from cell cultures for TCID<sub>50</sub> assays as described

471 previously [18]. Briefly, BHK-21 cells were seeded on a 96-well plate and grown to 60%

472 confluence. The supernatants were diluted in a 10-fold dilution series and added to each well

473 of the 96-well plate. One hundred microliters of each dilution was added in eight replicate

474 wells, and eight replicate mock controls were set. The plates were incubated at 37°C for 1.5 h.

475 Then the supernatants were discarded and replaced with 100 µL of DMEM supplemented

476 with 1% FBS. After 5 days in culture, the cytopathic effect was recorded. The TCID<sub>50</sub> of the

477 virus was calculated by Reed-Muench method [44].

478

479 **Indirect immunofluorescence and western blotting**

480 Indirect immunofluorescence and western blotting were performed as described

481 previously [18]. The antibodies used were mouse anti JEV E monoclonal antibody, rabbit anti

482 mosquito  $\beta$  actin polyclonal antibody, rabbit anti mosquito HSC70B polyclonal antibody,  
483 goat anti-mouse IgG-HRP antibody (1:10000; Santa Cruz), Alexa Fluor 405-conjugated  
484 anti-mouse IgG antibody (1:500; Abcam), Alexa Fluor 488-conjugated anti-rabbit IgG  
485 antibody (1:500; Thermo Fisher Scientific) and Alexa Fluor 594-conjugated anti-rabbit IgG  
486 antibody (1:500; Thermo Fisher Scientific). DAPI and DId were used for nucleus and  
487 membrane staining. Immunofluorescence was imaged with a Nikon C1Si confocal laser  
488 scanning microscope.

489 For tissue immunofluorescence assays, salivary glands were isolated on sialylated slides,  
490 washed with PBS, fixed with 4% paraformaldehyde for 1 h, and blocked in PBS with 2%  
491 bovine serum albumin (BSA) at room temperature for 2 h. The samples were incubated with  
492 mixture of JEV and *cpp* defensin A-FITC, detected with mouse anti JEV E monoclonal  
493 antibody and imaged with a Nikon C1Si confocal laser scanning microscope.

494

#### 495 **Protein expression and ELISA**

496 The purified JEV structural proteins (C, M, E, ED III) from the S2 insect expression  
497 system (Invitrogen) were quantified by using the bicinchoninic acid (BCA) assay. Expressed

498 proteins were used for ELISA or IFA analysis [47].

499 For defensin ELISA, defensin peptide was dissolved in PBS, then was diluted with 0.1

500 M dicarbonate (pH 9.6) to a final concentration of 250–750 ng. The plate was coated

501 overnight and incubated with 2% BSA for 2 h. Afterward, 100  $\mu$ L JEV virus ( $1 \times 10^5$  TCID<sub>50</sub>)

502 was added and incubated for 30 min at room temperature. The wells were washed with PBST

503 five times, mouse polyclonal antibody to JEV was added to the wells and incubated for 30

504 min. The wells were washed with PBST five times, and goat anti-mouse antibody labeled

505 with HRP was added. After incubation at room temperature 30 min and washing with PBST

506 five times, TMB was added to the wells as a chromogenic substrate. The plate was developed

507 in the dark for 10 min, and H<sub>2</sub>SO<sub>4</sub> was added to stop the reaction. The absorbance of each

508 well was read at 450 nm.

509 For viral protein ELISA, purified JEV structural proteins diluted in 0.1 M dicarbonate

510 (pH 9.6) were added to the plate wells. The plate was coated overnight and incubated with 2%

511 BSA for 2 h. Then 100  $\mu$ L defensin (50  $\mu$ M) labeled with FITC was added. The plate was

512 incubated for 30 min at room temperature and washed with PBST five times before

513 fluorescence measurement.

514 For C6/36 cell ELISA, the plates were pre-treated with polylysine. Healthy and fresh  
515 C6/36 cells were counted and diluted with 0.1 M dicarbonate (pH 9.6) to a final concentration  
516 of  $1 \times 10^5$  cells per well. The plate was processed as described above for JEV structural  
517 proteins or defensin coated ELISA.

518

519 **SILAC/MS analysis**

520 C6/36 cells were continuously passaged for eight generations on media with light,  
521 medium and heavy isotopes. All three labeling efficiencies reached 99%. The cells were  
522 grouped, inoculated with JEV or defensin, and collected at 24 h or 48 h according to the  
523 procedure. Equal amounts of cells from light, medium and heavy media in the same group  
524 were mixed to extract cell membrane proteins according to the manufacturer's instructions  
525 (Pierce). Extracted membrane proteins were quantified by BCA, identified by MS and  
526 normalized for further analysis.

527

528 **Statistical analysis**

529 All experiments were carried out in at least triplicate. Mean values  $\pm$  standard deviation

530 (SD) were calculated in Microsoft Excel. Statistical analysis was done with Student's t tests,

531 and values were considered significant when  $p < 0.05$ . Figures were created in GraphPad<sup>TM</sup>

532 Prism 5.0 software.

533

534 **Competing interests**

535 The authors declare no competing interests.

536

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542

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693

695 **Figure legends**

696 **Fig. 1. JEV infection induced defensin up-regulation in mosquito vectors.**

697 (A) JEV infection curve in mosquitoes. JEV ( $10^3$  MID<sub>50</sub>) or PBS was inoculated into female

698 mosquitoes by throat injection. Whole body, salivary gland and midgut samples were

699 collected at 4, 7 and 10 days post JEV infection. JEV E expression was quantified by

700 real-time PCR. (B) Expression levels of *cpp* defensins in the whole body. JEV ( $10^3$  MID<sub>50</sub>) or

701 PBS was inoculated into female mosquitoes by throat injection. *Cpp* defensin A mRNA levels

702 in the whole body at 7 and 10 days post JEV infection were quantified by real-time PCR. (C)

703 Expression levels of *cpp* defensins in the midgut and salivary gland. JEV ( $10^3$  MID<sub>50</sub>) or PBS

704 was inoculated into female mosquitoes by throat injection. Midgut and salivary gland were

705 separated at 7 days post infection. *Cpp* defensin A mRNA levels were quantified by real-time

706 PCR. (D) One step growth curve of JEV virus in C6/36. C6/36 cells were infected with 5

707 MOI and collected at different time points, as shown in Fig. 1D. JEV E mRNA levels were

708 quantified by real-time PCR. (E) Expression levels of *aa* defensins. C6/36 cells were infected

709 with 5 MOI and collected at 1, 3 and 5 days post JEV infection. *Aa* defensin C and total

710 defensins mRNA levels were quantified by real-time PCR. All experiments were done

711 in triplicate and were performed at least three times. Data are shown as Mean values  $\pm$

712 standard deviations.

713

714 **Fig. 2. Mosquito defensin facilitated JEV infection in mosquito vectors.**

715 (A) Mosquito defensin facilitated JEV infection in *culex* mosquitoes. Mosquito defensins

716 (100  $\mu$ M) and JEV (10 MID<sub>50</sub>) were pre-mixed at 4°C for 2 h and inoculated into female

717 mosquitoes. JEV E mRNA levels in the whole body at 7 and 10 days post JEV infection were

718 quantified by real-time PCR. (B) Mosquito defensins interference harmed JEV infection in

719 *culex* mosquitoes. Female mosquitoes were injected with siRNAs for 3 days, then JEV was

720 injected in dose of 10 MID<sub>50</sub>. JEV E mRNA levels in the whole body at 6 days post JEV

721 infection were quantified by real-time PCR. (C) Mosquito defensins facilitated JEV infection

722 on C6/36 in IFA detection. Mosquito defensins (50  $\mu$ M) and JEV (0.5 MOI) were pre-mixed

723 at 4°C and inoculated into C6/36 cells for 2 h (upper three panels). siRNAs target defensin

724 were transfected into C6/36 cell for 24 h, then JEV was inoculated to C6/36 cell for 2 h

725 (lower three panels). IFA was performed on cells at 3 days post infection. Bar, 10  $\mu$ m. (D)

726 Mosquito Defensins facilitated JEV infection in C6/36 cells. Mosquito defensins (50  $\mu$ M) and

727 JEV (0.5 MOI) were pre-mixed at 4°C and inoculated into C6/36 cells for 2 h. The cells and  
728 supernatant were collected at 3 days post infection to quantify JEV E mRNA levels (i) and  
729 TCID<sub>50</sub> (ii). (E) Defensin knockdown harmed JEV infection in C6/36 cell. siRNAs target  
730 defensin were transfected into C6/36 cell for 24 h, then equal JEV was added to C6/36 cell  
731 without changing media. The cells and supernatant were collected at 2 days post infection to  
732 quantify JEV E mRNA levels (i) and TCID<sub>50</sub> (ii). (F - G) Mosquito defensins inhibited JEV  
733 infection in mammalian cells. Mosquito defensins (50  $\mu$ M) and JEV (0.5 MOI) were  
734 pre-mixed at 4°C and were inoculated into Vero (F) or BHK (G) for 1.5 h at 37°C. The cells  
735 and supernatant were collected at 48 h post infection to quantify JEV E mRNA levels (i) and  
736 TCID<sub>50</sub> (ii). All experiments were done in triplicate and were performed at least three times.  
737 Data are shown as Mean values  $\pm$  standard deviations.

738

739 **Fig. 3. Mosquito Defensin facilitated JEV adsorption to mosquito cells.**

740 (A) Step scan of JEV infection on C6/36 cell. Steps of JEV infection on C6/36 cell were  
741 analyzed by different treatments. For binding, virus (0.5 MOI) and defensin (50  $\mu$ M) were  
742 mixed and inoculated to C6/36 cell on ice for 4 h, washed with PBS for five times, cultured

743 for 48 h with new media. For uncoating, virus (0.5 MOI) was inoculated to C6/36 cell on ice

744 for 4 h, washed with PBS for five times. Fresh media with defensin (50  $\mu$ M) was added to cell

745 for 6 h. After incubation, cell was washed and cultured for another 42 h with new media. For

746 replication, virus (0.5 MOI) was inoculated to C6/36 cell on ice for 4 h, washed with PBS for

747 five times. New media without defensin was added to cell for 48 h, and defensin (50  $\mu$ M) was

748 added into media at 6 h post culture. The cells were collected to quantify JEV E mRNA levels

749 by real-time PCR. (B) *Aa* defensin C facilitated JEV adsorption to C6/36 in a time-dependent

750 manner. *Aa* defensin C (50  $\mu$ M) and JEV (0.5 MOI) were pre-mixed at 4°C and inoculated

751 into C6/36 cells on ice for 4 h. Unabsorbed JEV was removed by washing with PBS three

752 times. The cells were collected to quantify JEV E mRNA levels by real-time PCR. (C and D)

753 IFA assay of JEV adsorption to C6/36 cells. *Aa* defensin C-FITC (50  $\mu$ M) and JEV (1 MOI)

754 were pre-mixed at 4°C and inoculated into C6/36 cells on ice. Unabsorbed JEV and defensin

755 were removed by washing with PBS three times. The cells were strained with antibody and

756 DAPI (C) or Did (D). (E) Co-localization of defensin and JEV on the cell surface. *Aa*

757 defensin C-FITC (50  $\mu$ M) and JEV (1 MOI) were pre-mixed at 4°C and inoculated into C6/36

758 cells on ice for 2 h, 4 h and 6 h. Unabsorbed JEV and defensin were removed by washing

759 with PBS three times. The cells were treated to observe JEV E (red fluorescence),

760 defensin-FITC (green fluorescence) and nuclei (blue fluorescence). Bar, 10  $\mu$ m. (F)

761 Co-localization of *Cpp* defensin A-FITC and JEV on the salivary gland. The salivary glands

762 from uninfected female mosquitoes were freshly isolated. Pre-mixed *cpp* defensin A-FITC

763 (50  $\mu$ M) and JEV (1 MOI) were added to salivary glands and incubated at room temperature

764 for 1 h. JEV E was labeled with monoclonal antibody (red fluorescence). Defensin-FITC was

765 detected by green fluorescence, and nuclei were stained with DAPI (blue fluorescence). Bar,

766 20  $\mu$ m. (G) JEV bind to defensin. The plate was coated with *aa* defensin C, incubated with

767 JEV and assessed with anti-JEV monoclonal antibody. (H) Defensin and JEV mixture binds

768 to C6/36 cells. The plate after polylysine treatment was coated with C6/36, pre-mixed

769 defensin and JEV were added, and detection was performed with anti-JEV antibody. (I)

770 Defensin binds to C6/36 directly. The polylysine treated plate was coated with C6/36,

771 defensin-FITC were added, and fluorescence value was detected. All experiments were done

772 in triplicate and were performed at least three times. Data are shown as Mean values  $\pm$

773 standard deviations.

774

775 **Fig. 4. Mosquito defensin bound JEV virions.**

776 (A) Viral proteins bind to mosquito defensin. The plate was coated with *aa* defensin C, and  
777 incubated with JEV structural proteins. Rabbit polyclonal antibodies to C protein and mouse  
778 monoclonal antibody to prM, E and ED III protein were utilized for viral protein binding  
779 detection. (B) Mosquito defensin-FITC bind to viral proteins. The plate was coated with  
780 purified JEV structural proteins, and incubated with *aa* defensin C-FITC. The fluorescence  
781 value of each well was measured. (C and D) Colocalization between defensin and E or ED III  
782 proteins. Defensin-FITC and E or ED III were pre-mixed at 4°C and inoculation into C6/36  
783 cells on ice for 4 h. Unabsorbed defensin and proteins were removed by washing with PBS  
784 three times. (C) The cells were stained with monoclonal antibody and DAPI to observe JEV E  
785 (red fluorescence), defensin-FITC (green fluorescence) and nuclei (blue fluorescence). (D)  
786 The cells were stained with monoclonal antibody and Did to observe JEV E (cyan  
787 fluorescence), defensin-FITC (green fluorescence) and membrane (red fluorescence). Bar, 10  
788 µm. All experiments were done in triplicate and were performed at least three times. Data are  
789 shown as Mean values ± standard deviations.

790

791 **Fig. 5. LRP2/defensin pathway mediates JEV adsorption.**

792 (A and B) Defensin adsorption was influenced by LRP2. The polylysine treated plate was

793 coated with C6/36, LRP2 siRNAs were transfected into cell. Defensin-FITC was inoculated

794 into cells at 24 h post transfection. After incubation on ice for 2 h, unabsorbed defensin was

795 removed by washing with PBS three times. Fluorescence value was detected by fluorescence

796 analyzer (A) or fluorescence microscope (B). (C, D and E) JEV adsorption on C6/36 cell was

797 influenced by LRP2/defensin pathway. The polylysine treated plate was coated with C6/36,

798 LRP2 siRNAs were transfected into cell. Pre-mixed JEV and *aa* Defensin C was inoculated

799 into cells at 24 h post siRNA transfection. After incubation at room temperature or on ice for

800 2 h, unabsorbed defensin and virus were removed by washing with PBS three times. For

801 real-time PCR (C) and TCID<sub>50</sub> (D) measurement, C6/36 cell and supernatant were collected at

802 2 days post infection. For IFA assay, C6/36 cell was treated immediately after inoculation on

803 ice (E). JEV E was labeled with monoclonal antibody (red fluorescence). Defensin-FITC was

804 detected by green fluorescence, and nuclei were stained with DAPI (blue fluorescence). Bar,

805 10  $\mu$ m. (F) *In vivo* JEV adsorption was influenced by LRP2/defensin pathway. Three days

806 after mosquitoes were injected with LRP2 siRNA, the mosquitoes were injected with

807 pre-mixed JEV and defensin. 6 days post infection, mosquitoes were collected to detect JEV

808 E mRNA levels in whole body. All experiments were done

809 in triplicate and were performed at least three times. Data are shown as Mean values  $\pm$

810 standard deviations.

811

812 **Fig. 6. Defensin down-regulated HSC70B on the C6/36 cell surface to enhance JEV**

813 **adsorption.**

814 (A) SILAC/MS workflow. (B) LC-MS/MS intensity of HSC70B on the C6/36 cell surface.

815 Intensity of HSC70B on cell surface was calculate. Protein levels were normalized in a mass

816 spectrometry computing program. (C) Validation of HSC70B expression on C6/36 cell

817 surface according to SILAC/MS. Mosquito HSC70B was probed by rabbit

818 polyclonal anti-HSC70B antibody. (D) The efficiency of HSC70B RNAi *in vitro*. HSC70B

819 siRNA target *aa* HSC70B was transfected into C6/36 cells for 24 h. Cell was collected and

820 HSC70B mRNA was measured by real-time PCR. (E) HSC70B interference facilitated JEV

821 adsorption to cells. C6/36 cells were inoculated with HSC70B siRNA for 24 h and then

822 inoculated with JEV or JEV and defensin on ice for 4 h. Unabsorbed JEV or defensin was

823 removed by washing with PBS three times. The cells were collected to quantify JEV E  
824 mRNA levels by real-time PCR. (F) The efficiencies of RNAi HSC70B *in vivo*. siRNA target  
825 *cpp* HSC70B was injected. The mosquitoes were collected at 3 days post injection to detect  
826 HSC70B mRNA levels *in vivo*. (G) HSC70B interference facilitated JEV infection *in vivo*.  
827 Three days after mosquitoes *in vivo* HSC70B RNAi, the mosquitoes were injected with 10  
828 MID<sub>50</sub> JEV. Mosquito samples were collected at 6 days post infection, and JEV E mRNA  
829 levels were detected by real-time PCR. All experiments were done  
830 in triplicate and were performed at least three times. Data are shown as Mean values ±  
831 standard deviations.

832

833 **Fig. 7. Mosquito defensin enhanced JEV replication in salivary gland**

834 (A) JEV E mRNA levels within salivary gland based on microinjection. *Cpp* defensin A (100  
835 μM) and JEV (10 MID<sub>50</sub>) were pre-mixed at 4°C for 2 h and injected into female mosquitoes.  
836 Salivary glands were isolated at 7 and 10 days post injection and detected by real-time PCR.  
837 (B) JEV E mRNA levels within salivary gland based on blood meal. *Cpp* defensin A (100  
838 μM) and JEV (10<sup>3</sup> MID<sub>50</sub>) were pre-mixed at 4°C. Mixture was added into fresh blood with

839 anticoagulant. Blood meal was performed for 2 h. Salivary glands were isolated at 7 and 10  
840 days post infection and detected by real-time PCR. All experiments were done  
841 in triplicate and were performed at least three times. Data are shown as Mean values ±  
842 standard deviations.

843

844 **Supplement Figures**

845 **Fig. S1. Sequence and abundance of mosquito defensins.**

846 (A) Amplification of *cpp* defensin A by PCR. (B) Defensin sequence alignment. Alignment  
847 of *cpp* defensin sequences (*Cpp* defensin A and unnamed defensin). (C) Abundance of  
848 defensins in C6/36 and *cpp*. Defensin genes were amplified and cloned into pMD18 plasmids,  
849 and positive plasmids were used to construct standard curves. Defensin abundance in cells or  
850 mosquitoes was quantified with a standard curve through real-time PCR. Defensin abundance  
851 is shown as a proportion. Target defensins are shown in gray in columns; the total column  
852 represents total defensins. (D, E) Defensin sequence alignment. Alignment of defensins in  
853 different mosquito Species (D). Alignment of mosquito defensins and human defensin (E).  
854 Alignment was performed by DNAMAN software. Data are shown as Mean values ±

855 standard deviations.

856

857 **Fig. S2. Major sequences used in this study.**

858 (A) *Culex pipiens pallens* defensin A protein sequence (NCBI number MH756645); the

859 mature defensin sequence is in red. (B) *Culex pipiens pallens* HSC70B partial sequence. (C)

860 *Culex pipiens pallens* unnamed defensin partial sequence. (D) C6/36 HSC70B protein

861 sequence (immunogenic peptide for antibody preparation is in red).

862

863 **Fig. S3. RNA interference efficiency in *in vitro* and *in vivo*.**

864 (A) The efficiency of defensins RNAi *in vivo*. siRNAs target *cpp* total defensins or defensin

865 A were injected. Mosquitoes were collected at 3 days post injection to detect defensins

866 mRNA levels by real-time PCR. (B) The efficiency of defensins RNAi *in vitro*. siRNAs target

867 *aa* defensins was transfected into C6/36 cells for 24 h. Cell was collected and defensins

868 mRNA were measured by real-time PCR. (C) The efficiency of LRP2 RNAi *in vitro*. LRP2

869 siRNA target *aa* LRP2 was transfected into C6/36 cells for 24 h. Cell was collected and LRP2

870 mRNA was measured by real-time PCR. (D) The efficiency of LRP2 RNAi *in vivo*. siRNA

871 target *cpp* LRP2 was injected. Mosquitoes were collected at 3 days post injection to detect  
872 LRP2 mRNA levels by real-time PCR. All experiments were done  
873 in triplicate and were performed for three times. Data are shown as Mean values  $\pm$  standard  
874 deviations.

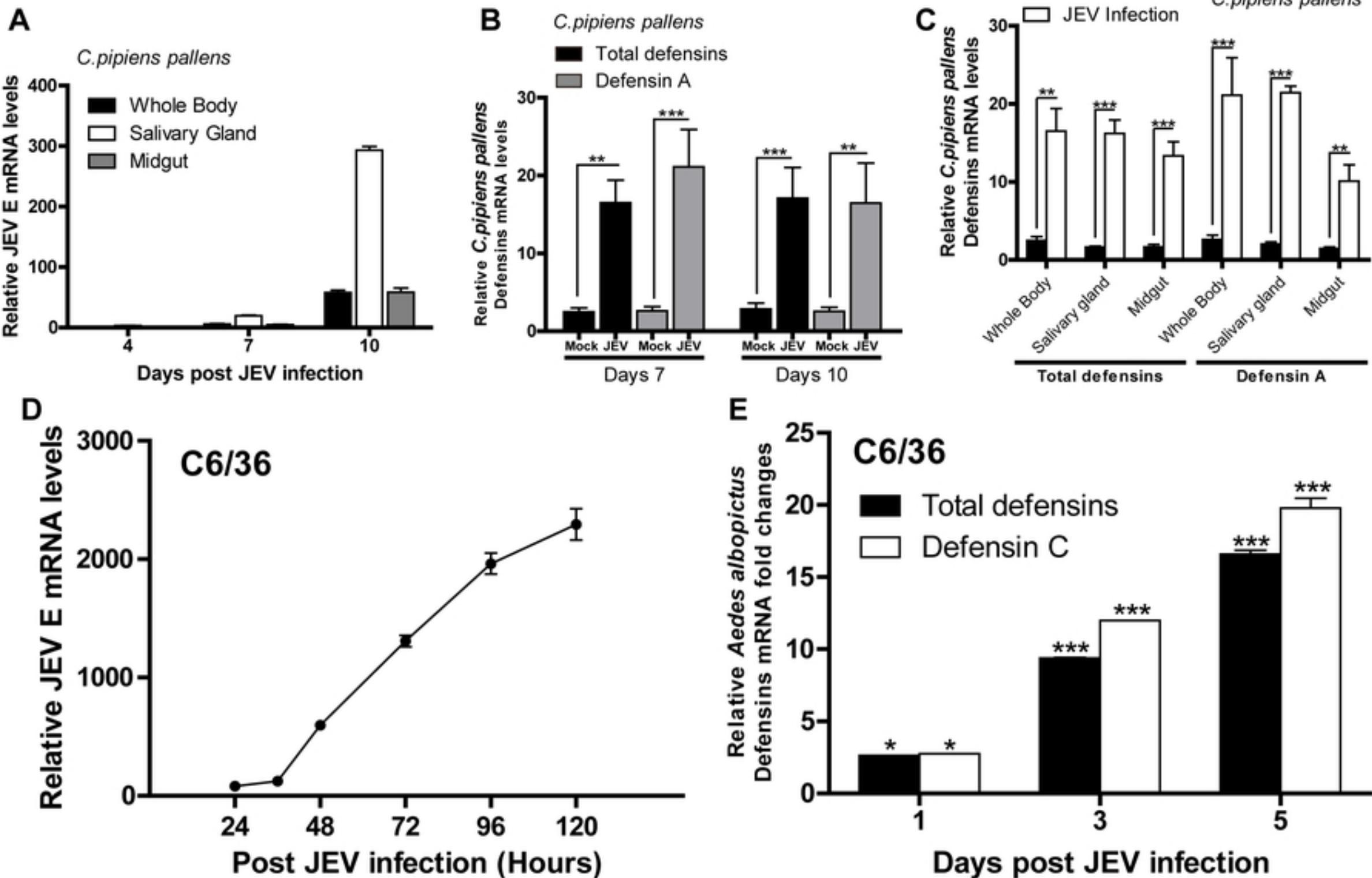


Figure 1

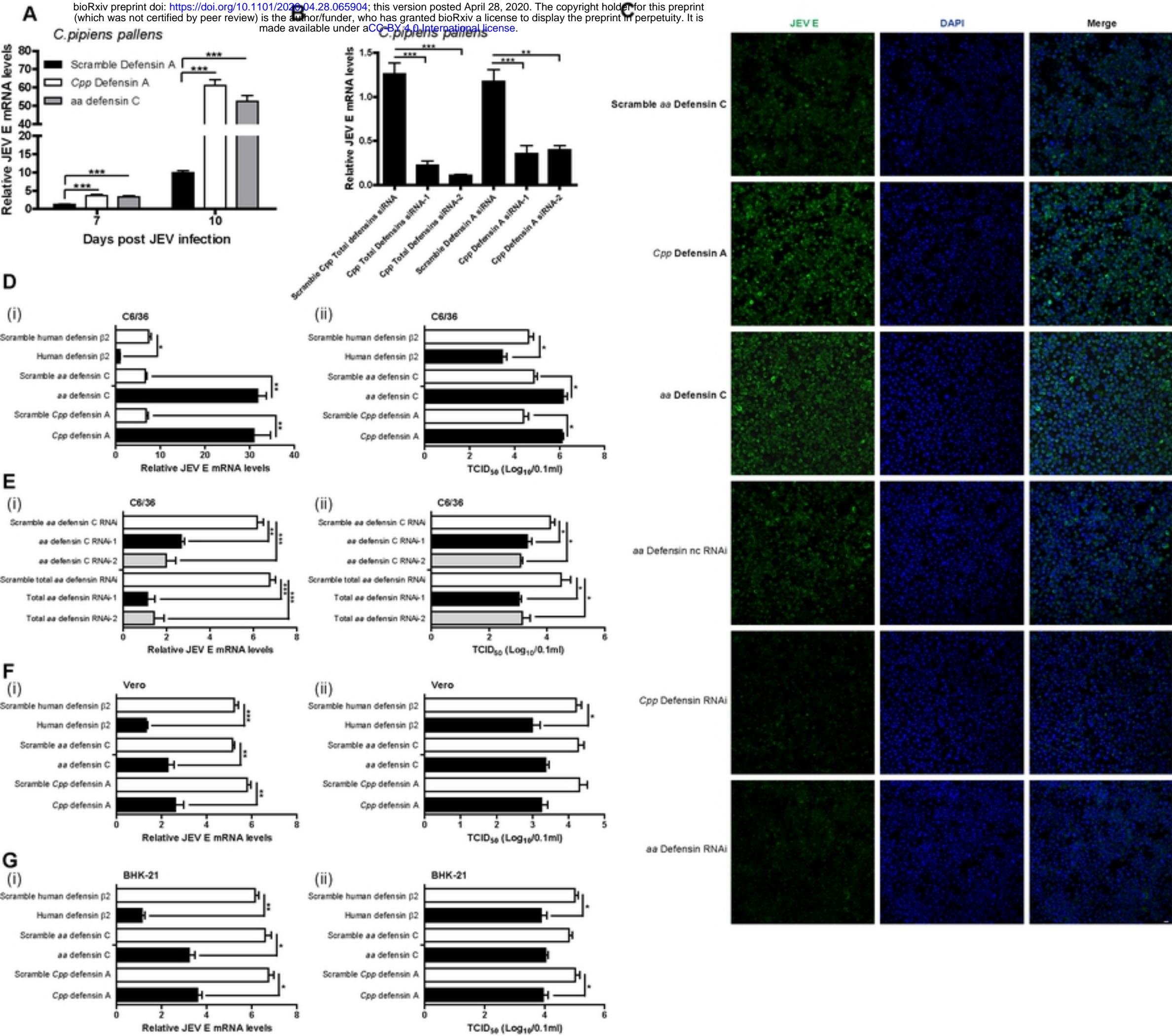


Figure 2

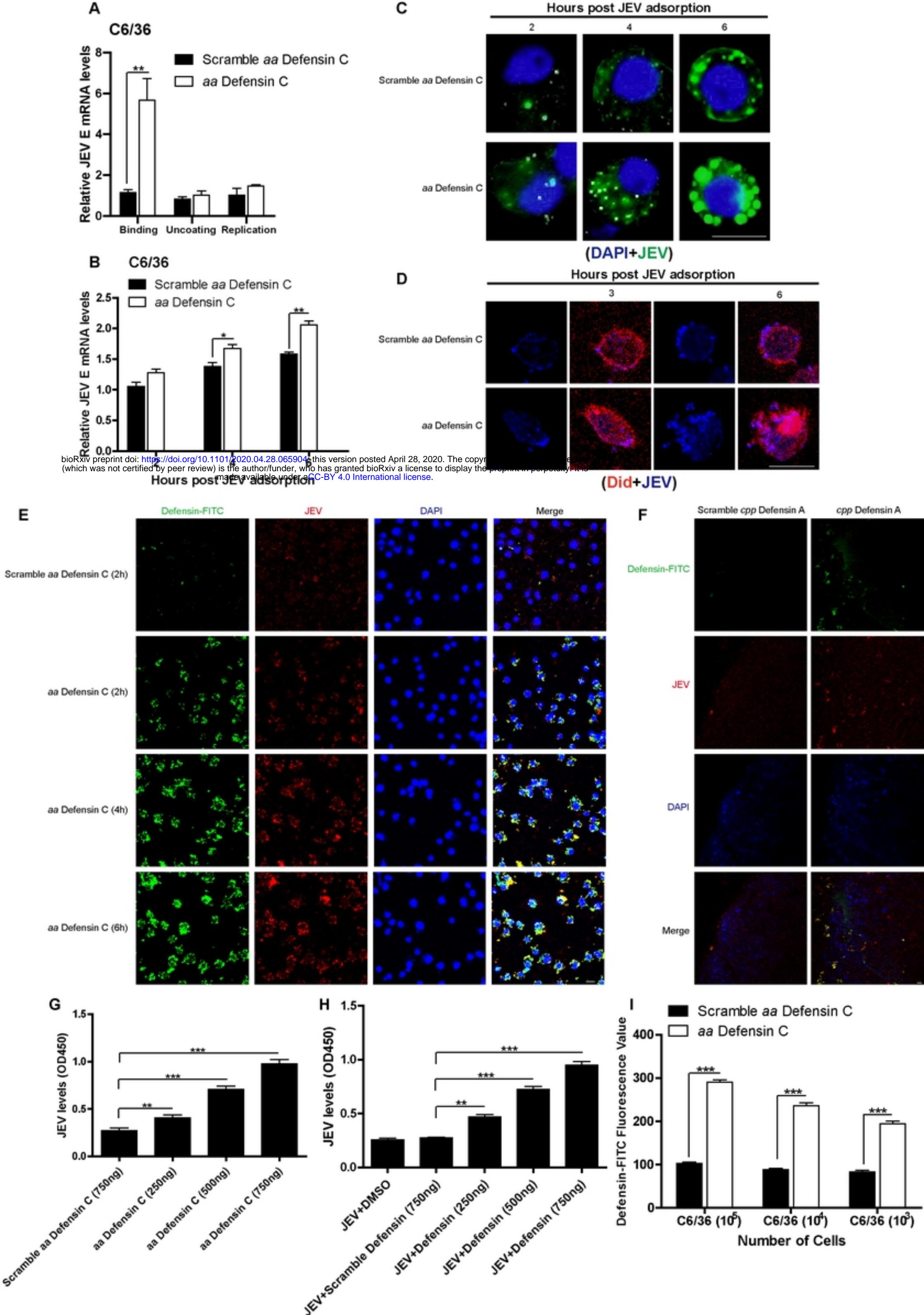


Figure 3

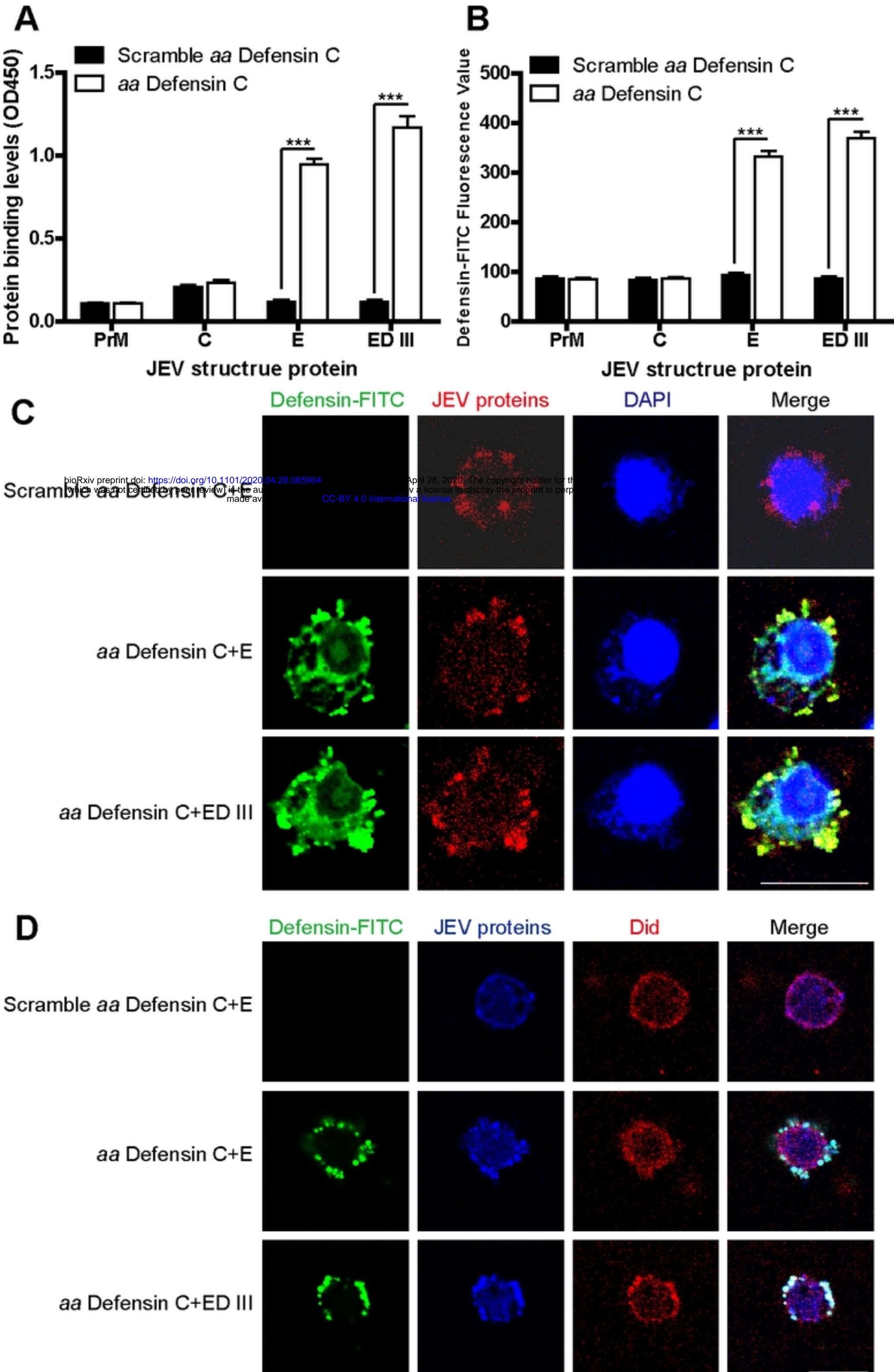


Figure 4

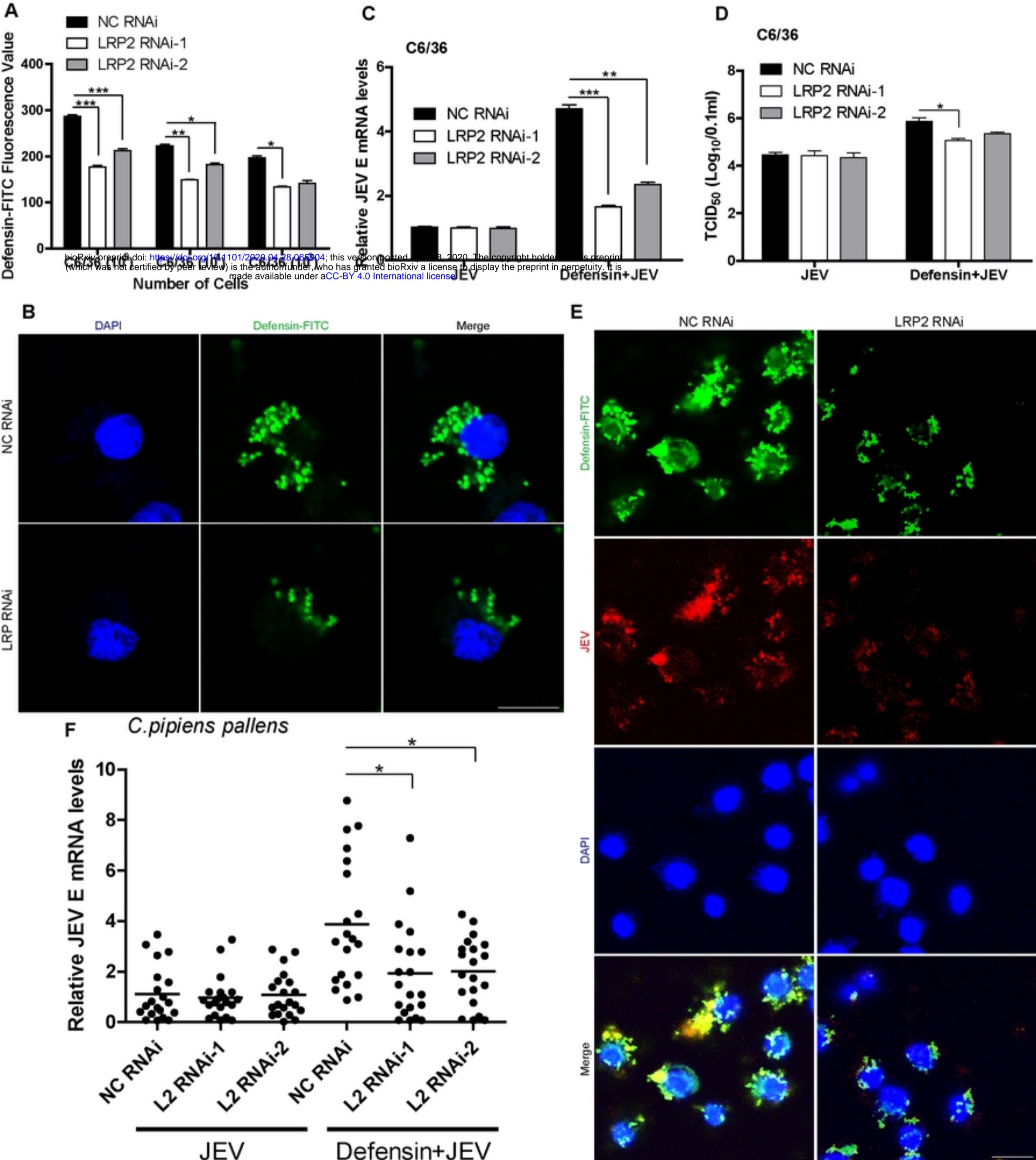
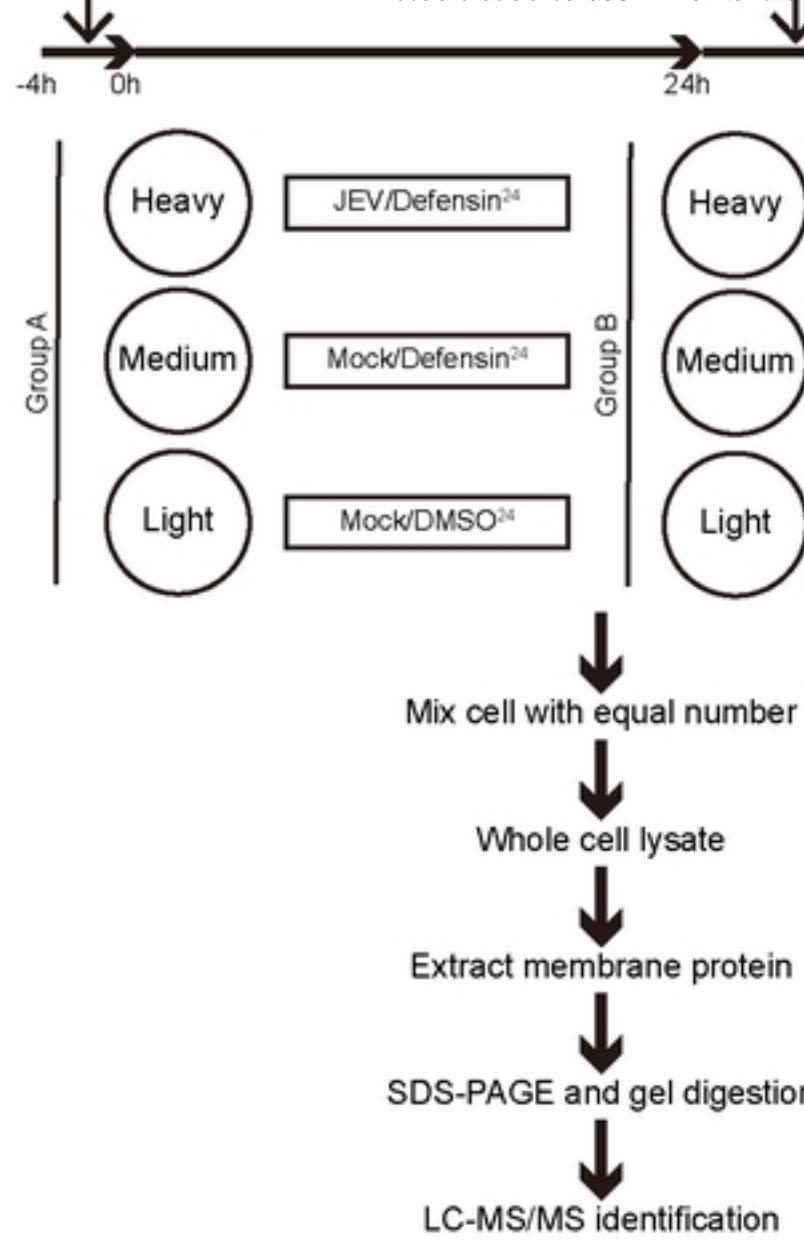


Figure 5



### LC-MS/MS intensity

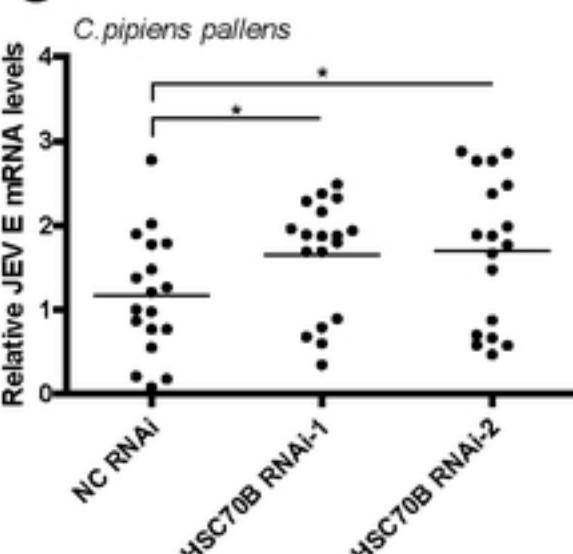
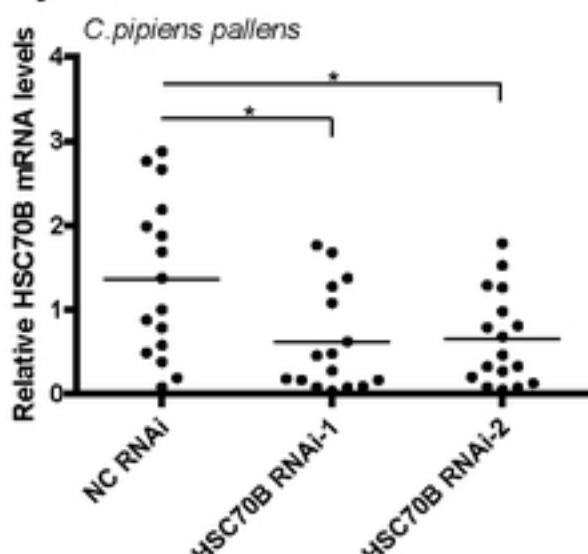
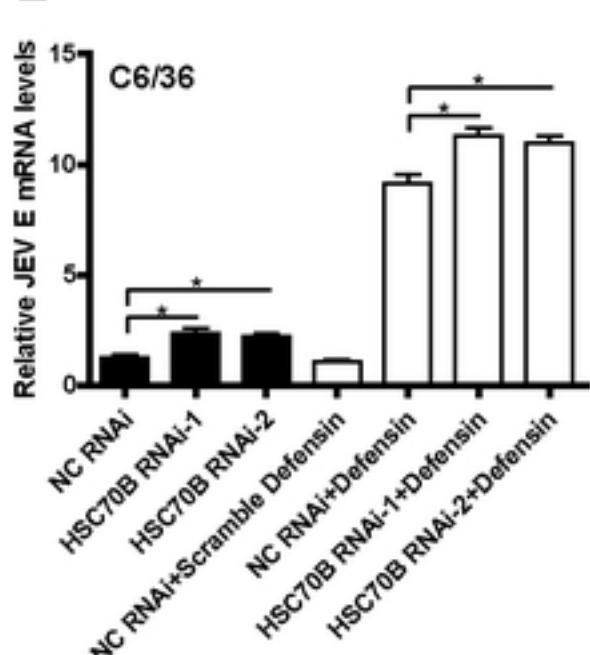
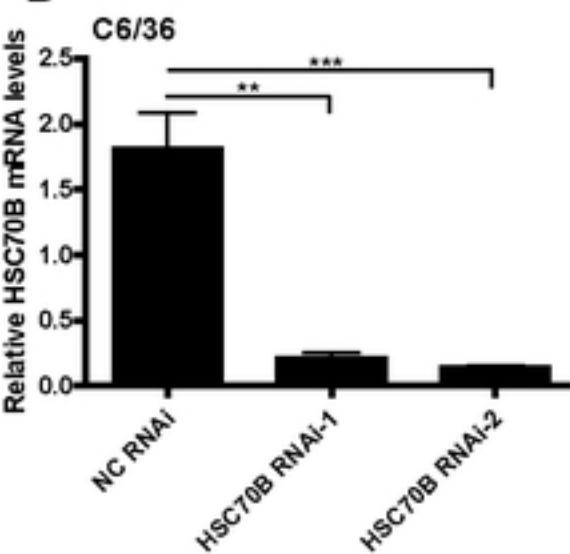
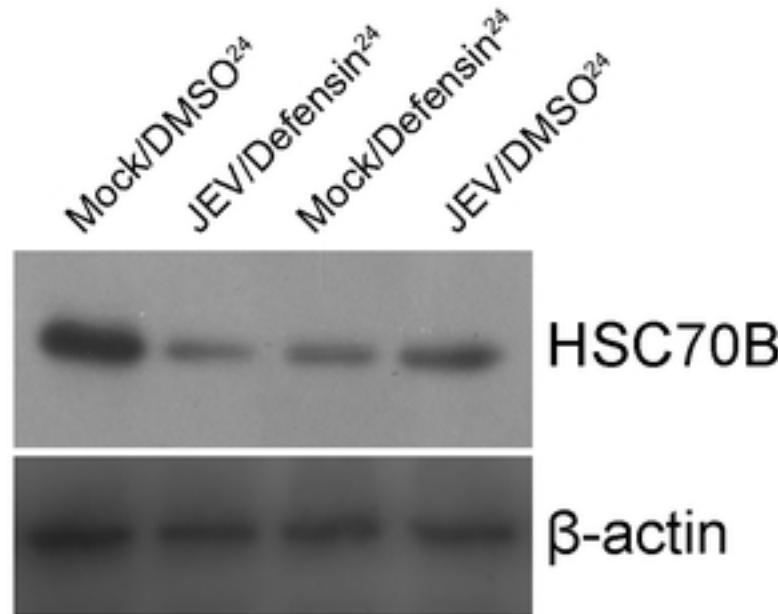
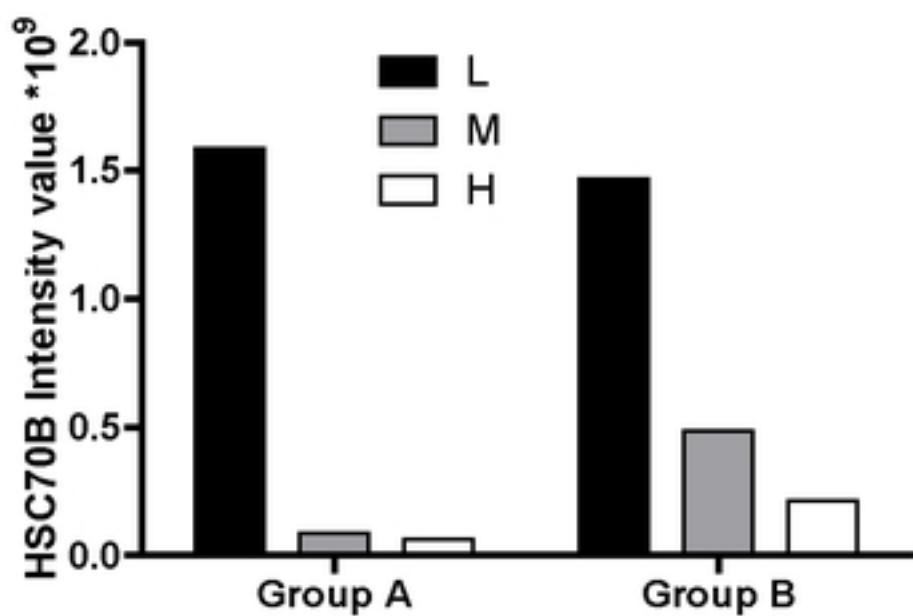


Figure 6

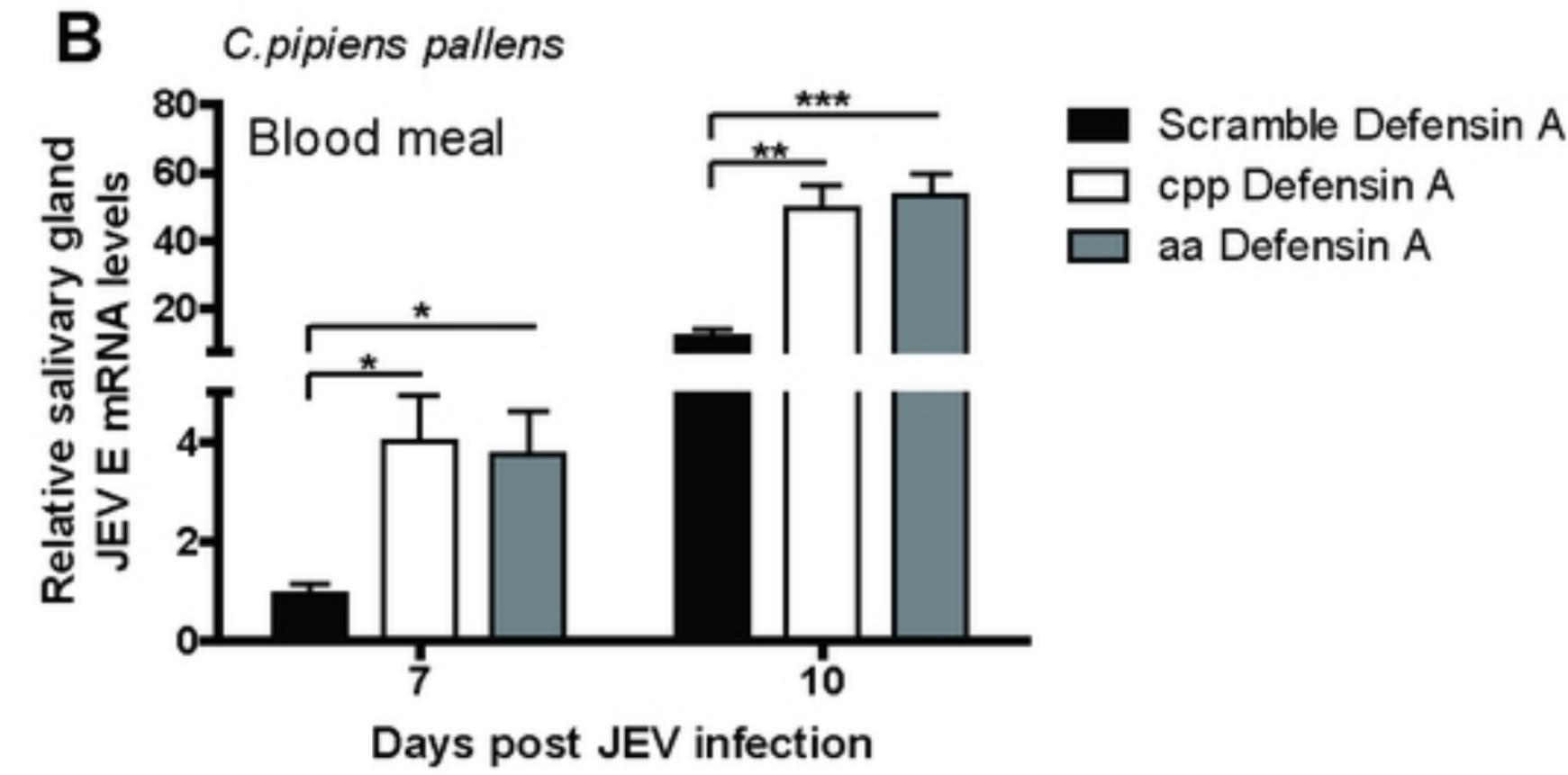
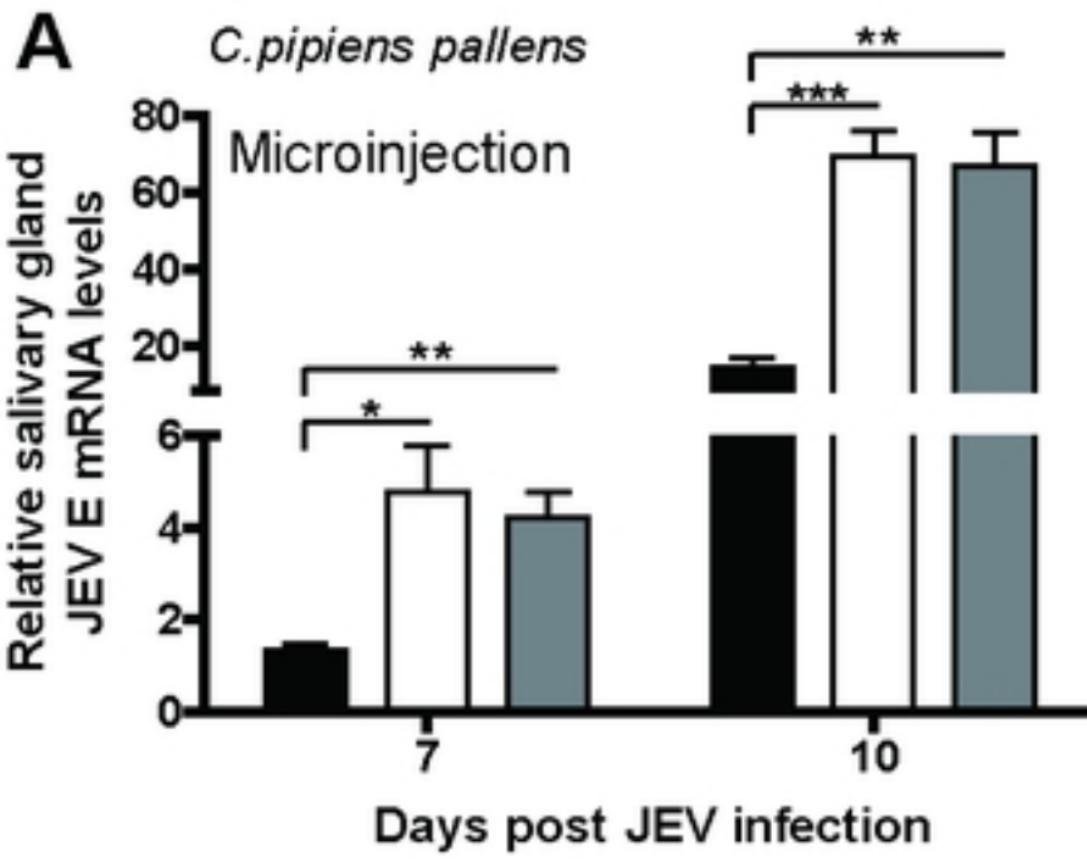


Figure 7