bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

1 **Title page**

2

3 # miR-2 contributes to WSSV infection by targeting Caspase 2 in
4 # mud crab (*Scylla paramamosain*)

5

6 Yi Gong[1,2], Jiao Chen[1,2], Yalei Cui[3], Shengkang Li[1,2*]

7

8 [1]*Guangdong Provincial Key Laboratory of Marine Biology, Shantou University,*
9 *Shantou 515063, China*
10 [2] *Institute of Marine Sciences, Shantou University, Shantou 515063, China*
11 [3]*College of Animal Science and Technology, Henan Agricultural University, Zhengzhou*
12 *450002, China*

13

14 * Corresponding author: Shengkang Li
15 Tel: +86-754-86502485
16 Fax: +86-754-86503473
17 Email: lisk@stu.edu.cn

18

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

19 **Summary**

20 As we known, Caspase 2 is widely studied for its apoptosis regulatory function in
21 mammals. However, despite the fundamental role of apoptosis during the anti-viral
22 immune response, the relationship between Caspase 2 and virus infection has not been
23 extensively explored in invertebrates, whether miRNAs are involved in this process also
24 remains unclear. To address this issue, the miRNA-mediated regulation of Caspase 2 in
25 mud crab *Scylla paramamosain* was characterized in this study. The results suggested
26 that *Sp*-Caspase 2 could suppress white spot syndrome virus (WSSV) infection via
27 apoptosis induction. The further data showed that Caspase 2 was directly targeted by
28 miR-2 in mud crab. Silencing or overexpression of miR-2 could affect apoptosis and
29 WSSV replication through regulating the expression level of Caspase 2. Taken together,
30 all these results demonstrated the crucial role of miR-2-Caspase 2 pathway in the innate
31 immunity of mud crab and revealed a novel mechanism during anti-viral immune
32 response in marine invertebrates.

33

34 **Keywords:** Caspase 2; miR-2; WSSV; apoptosis; *Scylla paramamosain*

35

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

36 **Introduction**

37 It is well known that crustaceans lack adaptive immunity and mainly rely on innate
38 immune system (including humoral and cellular immunity) to recognize and protect the
39 host against harmful microbes [1, 2]. In the recent years, growing evidence have
40 demonstrated that the alterations associated with cell survival contribute to the
41 pathogenesis of many diseases, including viral infections and autoimmune diseases [3].
42 Apoptosis, which is also called cell programmed death with specific features, including
43 cytoplasmic narrow, membrane blebbing and chromatin aggregation [4], can be
44 activated by various physiological and pathological stimulation [5]. It has been reported
45 that virus infection would cause apoptosis activation in host [6], which could also serve
46 as an effective defensive approach of the host cells to suppress virus invasion [7].
47 However, at present, the involvement of apoptosis during anti-viral immune response
48 have not been extensively explored in marine invertebrates.

49 As we know, the caspase members are required for apoptosis induction and signal
50 amplification [8]. Among them, as the second found caspase member, Caspase 2 is the
51 most evolutionarily conserved in animals [9]. The gene for Caspase 2 was initially
52 termed Nedd2 (neuronally expressed developmentally downregulated gene 2) during
53 hybridization screening for mouse genes in neural precursor cells [10]. After that,
54 Nedd2 was shown to be homologous to the *C. elegans* death gene-3 (CED-3) [11]. It
55 has been reported that Caspase 2 is located in Golgi, mitochondria, nuclei and
56 cytoplasm, and could serve as the upstream of DNA damage-induced apoptosis
57 pathway by promoting cytochrome c release from mitochondria [12]. Moreover,

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

58 cytotoxic stress could cause activation of Caspase 2, which is required for the
59 permeabilization of mitochondria [13]. So far, Caspase 2-associated researches are
60 mainly focused on higher animals, besides, the correlation with antiviral immune
61 regulation also has not been addressed.

62 MicroRNAs (miRNAs), approximately 21~27 nucleotide (nt) in length [14], are
63 endogenous small non-coding RNA molecules that could bind to the 3'UTRs of target
64 mRNAs by seed sequence complementary pairing, which further downregulate the
65 expressions of target genes through translation repression or direct mRNA degradation
66 [15]. It has been reported that miR-383 could promote human epithelial ovarian cancer
67 development by inhibiting the expression of Caspase 2 [16]. Additionally, miR-708
68 could act as an oncogene and induce the carcinogenicity of bladder cancer by down-
69 regulating Caspase 2 level [17]. Thus, it can be concluded that Caspase 2 was able to
70 be regulated by miRNAs in various biological process. However, most of the researches
71 associated with Caspase 2 targeted by miRNAs are mainly focused on cancer at present,
72 whether Caspase 2 can be regulated by miRNAs and its roles has not been intensively
73 investigated in invertebrates. To address this issue, the miRNA targeting Caspase 2 in
74 mud crab was characterized in our study. White spot syndrome virus (WSSV), a large
75 enveloped double-stranded DNA viral pathogen for many marine crustaceans [18], was
76 used as an infection model. The results of this study revealed that miR-2 could affect
77 apoptosis and WSSV replication through regulating the expression level of Caspase 2,
78 indicating the crucial role of miR-2-Caspase2 pathway during anti-viral immune
79 response of mud crab.

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

80 **Results**

81 **Bioinformatics analysis of *Sp*-Caspase 2 cDNA sequence**

82 The complete cDNA sequence of *Sp*-Caspase 2 contains an open reading frame
83 (ORF) of 969 bp encoding 322 deduced amino acids, the sequence has been deposited
84 in GenBank under the accession number MH558571.1. The putative protein sequence
85 of *Sp*-Caspase 2 possesses a conserved CASc domain (amino acids 72-320) (Fig. 1A).
86 Besides, the tertiary structure of *Sp*-Caspase 2 was predicted and the active sites were
87 marked (Fig. 1B). Besides, the amino acid sequence of *Sp*-Caspase 2 was aligned with
88 other crustaceans, the results showed that *Sp*-Caspase2 display the highest identity with
89 *Pt*-Caspase 2 (92%) from *P. trituberculatus* (ARO92228.1) (Fig. 1C). Moreover,
90 multiple sequence alignment indicated that the CASc domain of Caspase 2 was
91 conserved across the species (Fig. 1C).

92 **Role of Caspase2 on virus infection in mud crab**

93 To examine the effect of Caspase 2 on virus infection, we detected the expression
94 level of Caspase 2 in mud crab challenged with WSSV. The results revealed that both
95 mRNA and protein levels of Caspase 2 were upregulated during WSSV infection
96 (Fig.2A and 2B), indicating that Caspase 2 may participate in the immune response to
97 virus. To further ascertain whether Caspase 2 could affect WSSV proliferation in mud
98 crab, Caspase 2 was silenced and then WSSV replication was evaluated. The results
99 showed that the protein level of Caspase 2 was extremely decreased in mud crab after
100 treated with Caspase 2-siRNA compared to control group (Fig. 2C). Besides, it was
101 found that silence of Caspase 2-siRNA could suppress the expression of viral gene

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

102 VP28 during WSSV infection (Fig. 2D). Taken together, these data strongly suggested
103 that Caspase 2 is involved in immune response to virus infection, and could suppress
104 virus proliferation in mud crab.

105 **Effects of Caspase 2 in regulating apoptosis of mud crab**

106 To further explore how Caspase 2 participate in the resistance to WSSV infection
107 in mud crab, we attempt to detect Caspase 3/7 activity and apoptosis rate in the mud
108 crabs treated with either WSSV or Caspase 2-siRNA. The results showed that both
109 Caspase 3/7 activity and apoptotic rate of mud crab hemocytes were significantly
110 increased following WSSV challenge compared with the control (Fig. 3A and 3B),
111 indicating that virus infection could induce apoptosis in mud crab. Moreover, we found
112 that the Caspase 3/7 activity and apoptotic rate of hemocytes were all decreased in mud
113 crabs treated with WSSV and Caspase 2-siRNA compared to such in mud crabs treated
114 with WSSV only (Fig. 3A and 3B). All these results demonstrated that Caspase 2 could
115 protect mud crab from WSSV infection via promoting apoptosis.

116 **The interactions between Caspase 2 and miR-2 in mud crab**

117 To reveal the mechanism of Caspase 2 upregulation during virus infection in mud
118 crab, miRNA targeting Caspase 2 were predicted using bioinformatics. The results
119 showed that Caspase 2 could be potentially targeted by miR-2 (Fig. 4A). Then, to
120 characterize the interaction between Caspase 2 and miR-2, the plasmid EGFP-Caspase
121 consisting of EGFP and Caspase 2 3’UTR was constructed, and EGFP-Δ Caspase 2
122 3’UTR was served as control (Fig. 4B). After that, miR-2 and the constructed plasmids
123 were co-transfected into S2 cells, the results showed that the fluorescence intensity of

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

124 S2 cells treated with the EGFP-Caspase 2 3'UTR and miR-2 was significantly
125 decreased compared with the controls, suggesting that miR-2 could bind with Caspase
126 2 3'UTR and inhibit its expression (Fig. 4C). Furthermore, we detected the subcellular
127 location of Caspase 2 mRNA and miR-2 in the hemocytes of mud crabs by fluorescence
128 in situ hybridization, the results indicated that miR-2 was co-localized with Caspase 2
129 mRNA (Fig. 4D).

130 To verify the effect of miR-2 on Caspase 2 expression, miR-2 was silenced or
131 overexpressed in mud crabs, followed by detection of Caspase 2 levels. The results
132 showed that both mRNA and protein levels of Caspase 2 were significantly increased
133 when miR-2 was silenced in mud crab (Fig. 5A and 5B), while decreased in the miR-2
134 overexpressed mud crabs compared with the control group (Fig. 5A and 5B). Taken
135 together, the above data suggested that miR-2 could directly interacted with Caspase 2
136 mRNA and suppress its expression.

137 **The involvement of miR-2-Caspase 2 pathway in response to WSSV infection**

138 In an attempt to explore the role of miR-2 during virus infection, we detected the
139 expression level of miR-2 during WSSV infection. The results of qPCR revealed that
140 WSSV infection resulted in a significant decrease of miR-2 expression in mud crab (Fig.
141 6A), suggesting that miR-2 might participate in immune regulation of mud crab. To
142 further evaluate the effect of miR-2 on virus infection, miR-2 was overexpressed or
143 silenced during WSSV infection, the results showed that the expression of viral gene
144 VP28 was significantly decreased in miR-2 silenced mud crab (Fig. 6B), while
145 upregulated when miR-2 was overexpressed compared with the controls (Fig. 6C).

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

146 Furthermore, when mud crab was co-treated with AMO-miR-2 and Caspase 2-siRNA,
147 the AMO-miR-2-mediated virus suppression was remarkably relieved. These results
148 revealed that miR-2 could promote WSSV infection by targeting Caspase 2 in mud
149 crabs.

150 **The effects of miR-2-Caspase 2 pathway on apoptosis regulation**

151 To explore the role of miR-2 during Caspase 2-mediated apoptosis regulation, the
152 apoptosis activity was evaluated in mud crabs treated with AMO-miR-2 and Caspase
153 2-siRNA. The results showed that both Caspase 3/7 activity and apoptosis rate of mud
154 crab hemocytes were significantly increased when miR-2 was silenced (Fig. 7A and
155 7B), suggesting that miR-2 was an anti-apoptotic miRNA in mud crab. Moreover, in
156 the mud crab co-treated with AMO-miR-2 and Caspase 2-siRNA, the upregulated
157 apoptosis activity in mud crab caused by miR-2 interference was significantly reduced,
158 indicating that miR-2 could suppress apoptosis by targeting Caspase 2 in mud crab.

159 In summary, the above data showed that the expression of miR-2 was significantly
160 decreased during WSSV infection in mud crab, resulting in the upregulation of Caspase
161 2 and the activation of cell apoptosis, which eventually suppressed virus replication in
162 mud crab (Figure 8).

163

164 **Discussion**

165 In the past few years, it has become clear that the host could prevent virus
166 replication and dissemination by apoptosis induction during infection process [19].
167 Shrimp miR-12 could simultaneously trigger phagocytosis and apoptosis of hemocytes

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

168 through the synchronous downregulation of PTEN (phosphatase and tensin homolog)
169 and TMBIM6 (transmembrane BAX inhibitor motif containing 6) in response to WSSV
170 infection [20]. Similarly, through virus-host co-evolution, viruses also developed
171 distinct strategies to overcome immunological defenses of the host by subverting host
172 cell apoptosis [21]. It was found that the early-expressed nonstructural proteins (NS1
173 and NS2) of respiratory syncytial virus (RSV) could promote virus replication by
174 delaying host cell apoptosis via IFN- and EGFR-independent pathway [22]. During
175 WSSV infection, the virus-encoded miRNA WSSV-miR-N24 could target host caspase
176 8 gene and further repress apoptosis of shrimp hemocytes, which enhanced WSSV
177 copies in shrimp [23]. At present, the relevant investigations performed in marine
178 invertebrates are quite limited. In our study, we found that the apoptosis level in mud
179 crabs was remarkably upregulated during WSSV infection, and demonstrated the
180 essential role of miR-2-Caspase 2 pathway in the regulation of apoptosis and virus
181 infection. Therefore, our findings revealed a novel miRNA-mediated regulatory
182 mechanism during antiviral immune response in mud crab.

183 The first described feature of Caspase 2 is the activator of extrinsic apoptosis
184 pathway [24]. The mitosis-promoting kinase, cdk1-cyclin B1 can phosphorylate
185 Caspase 2 at Ser 340 to prevent its activation, which further suppresses apoptosis
186 upstream of mitochondrial cytochrome c release [25]. In the recent years, Caspase-2
187 has also been proved to mediate non-apoptotic signaling pathways. It has been reported
188 that Caspase 2 could serve as a tumor suppressor in Kras (kirsten rat sarcoma viral
189 oncogene)-driven lung cancer, silencing of Caspase 2 would lead to enhanced tumor

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

190 proliferation and progression [26]. Also, Caspase 2 was found to form complexes with
191 the cell cycle regulatory proteins cyclin D3, CDK4, and p21/Cip1 to promote AR
192 (androgen receptor) transactivation by inhibiting the repressive function of cyclin D3
193 [27]. So far, Caspase 2 has been widely studied for its apoptotic and non-apoptotic
194 functions, however, the relationship between Caspase 2 and virus infection has not been
195 previously addressed. In this study, the involvement of Caspase 2 during WSSV
196 infection was determined, the results showed that both mRNA and protein levels of
197 Caspase 2 in mud crab was significantly upregulated after WSSV challenge, and the
198 copy numbers of WSSV was increased when Caspase 2 was silenced. For the first time,
199 the present study revealed the crucial role of Caspase 2 in the immune response to virus
200 infection in mud crab.

201 RNAi, mainly mediated by siRNAs or miRNAs, was a natural defensive approach
202 to virus infection through post-transcriptional gene regulation [28]. It has been proved
203 that the miRNA-mediated RNAi pathways were important regulators in many
204 biological processes [29]. Silencing of miR-100 would result in the increase of
205 apoptotic activity of shrimp hemocytes by regulating the expression of trypsin, which
206 further led to the decreases of virus genome copies during WSSV infection [30].
207 Besides, it was found that miR-200c could be induced by oxidative stress and caused
208 endothelial cell apoptosis and senescence by targeting ZEB1 (Zinc finger E-Box
209 binding homeobox 1) [31]. Previous researches have demonstrated that miRNAs were
210 tightly relevant to the regulation of apoptosis and immune regulation in both vertebrates
211 and invertebrates. However, whether Caspase 2 could be regulated by miRNAs and

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

212 their biological significances remains unaddressed in invertebrates. Here, we revealed
213 the transcriptional crosstalk between miR-2 and Caspase 2 during WSSV infection in
214 mud crab, and found that the miR-2 was downregulated after WSSV challenge, leading
215 to the accumulation of Caspase 2, which eventually triggered apoptosis and attenuated
216 WSSV replication in mud crab. In this context, our findings provided a clue to clarify
217 the role of miRNAs during Caspase 2-mediated apoptosis regulation and virus
218 suppression in marine invertebrates.

219

220 Materials and methods

221 Mud crab culture and WSSV challenge

222 Healthy mud crabs were purchased from Niutianyang farm (Shantou, Guangdong,
223 China) and acclimated in tanks under laboratory conditions (10 ‰ salinity, 25 ºC) for a
224 week before experiments. Then, each crab was injected with 200 μL of WSSV solution
225 (1×10^6 copies/mL) according to our previous study [32], PBS-treated mud crabs was
226 served as control group. At different times post-infection, hemocytes and muscles were
227 collected from three randomly chosen crabs per group and stored at -80 ºC for later use.

228 RNA interference of Caspase 2

229 The siRNA targeting mud crab Caspase 2 was designed with BLOCK-iT RNAi
230 Designer (https://rnaidesigner.lifetechnologies.com/rnaiexpress/sort.do) based on the
231 sequence of Caspase 2 (GenBank accession number MH558571.1), generating
232 Caspase2-siRNA (5’-UGUUACACGGUCAAAGUAGCGUU-3’) and its control
233 Caspase2-siRNA-scrambled (5’-AAGAGCGAUGGCGUAUACUUCUU-3’). The

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

234 siRNAs were synthesized using the *in vitro* transcription T7 kit (TaKaRa, Japan). Then,
235 50 μg of Caspase2-siRNA or Caspase2-siRNA-scrambled was injected into each mud
236 crab in two doses, at intervals of 12 h. At different time post the last injection, three
237 mud crab were randomly selected for each treatment at stored at -80 ºC for later use.

238 **Analysis of WSSV copies with qPCR**

239 The genomic DNA of WSSV was extracted from crab muscle with a SQ tissue
240 DNA kit (Omega Bio-tek, USA) according to manufacturer's instruction, the copy
241 number of WSSV was detected by qPCR analysis using Premix Ex Taq (Probe qPCR)
242 (TaKaRa, Japan). The qPCR was performed with WSSV-specific-primers (5'-
243 TTGGTTTCATGCCCGAGATT-3' and 5'-CCTTGGTCAGCCCCTTGA-3') and a
244 TaqMan probe (5'-FAM-TGCTGCCGTCTCCAA-TAMRA-3') as described in our
245 previous study [33].

246 **Quantification of mRNA with qPCR**

247 Total RNAs were extracted from crab hemocytes by RNA isolation kit (Ambion,
248 USA). Reverse transcription reaction was conducted with PrimeScript™ RT Reagent
249 Kit (Takara, Japan). The primers (5'-GGGACAAGGAACAACAGAAT-3' and 5'-
250 ACACGGTCAAAGTAGCGATG-3') were used to quantify the Caspase 2 mRNA, β-
251 actin was served as the internal control, which quantified with primers (5'-
252 GCGGCAGTGGTCATCTCCT-3' and 5'-GCCCTTCCTCACGCTATCCT-3'). Then,
253 the relative fold change of Caspase 2 expression levels was determined using the $2^{-\Delta\Delta Ct}$
254 algorithm.

255 **Bioinformatics analysis of Caspase2**

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

256 The comparison of Caspase2 full-length cDNA and deduced amino acid sequences
257 between different species was conducted by BLAST software at the National Center
258 for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/). The deduced
259 amino acid was obtained by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi),
260 then Signal P 3.0 program was used to predict the presence and location of signal
261 peptide (http://www.cbs.dtu.dk/services/SignalP). The transmembrane domain was
262 predicted by the TMHMM (http://www.cbs.dtu.dk/services/TMHMM). Besides, the
263 tertiary structure of Caspase 2 protein was designed online and edited by PyMol Viewer
264 (https://zhanglab.ccmb.med.umich.edu/I-TASSER).

265 **Detection of apoptotic activity**

266 To detect apoptosis rate, the hemocytes of crabs were collected by centrifugation
267 at 600×g for 10 mins at 4 ºC, then, the hemocytes were washed twice and treated
268 according to protocol of BD Pharmingen FITC Annexin V Apoptosis Detection Kit (BD
269 Biosciences, US). In addition, the hemocytes were also placed onto a 96-well plate at a
270 density of 10^5 hemocytes per well, after added 100 mL of Caspase-Glo 3/7 reagent
271 (Promega, USA), the plate was gently shaken at room temperature for 2 h and the
272 luminescence was measured by a plate-reading luminometer.

273 **Cell culture, transfection, and fluorescence assays**

274 The Drosophila Schneider 2 (S2) cells (Invitrogen) were co-transfected with the
275 EGFP-Caspase 2 or EGFP-Δ Caspase 2 plasmid and the synthesized miR-2 (miR-2-
276 mimic) using Cellfectin II transfection reagent (Invitrogen) according to the
277 manufacturer's protocol. The concentrations of miR-2-mimic and the plasmid were 50

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

278 nM/well and 100 ng/well, respectively. At 48 h after co-transfection, the EGFP
279 fluorescence intensity was measured by microplate reader at 490/ 510 nm of
280 excitation/emission (Ex/Em).

281 **Fluorescence in situ hybridization**

282 The hemocytes were seeded onto polysine-coated coverslips and then fixed with
283 4% polyformaldehyde for 15 min at room temperature. Followed by dehydrated in 70%
284 ethanol at 4 °C overnight. After that, the coverslips were incubated with hybridization
285 buffer [1× SSC (15 mM sodium citrate, 150 mM sodium chloride, pH 7.5), 10% (w/v)
286 dextran sulfate, 25% (w/v) formamide, 1× Denhardt's solution] containing 100 nM of
287 probe at 37 °C for 5 h. The probe used is listed below, miR-2 probe (5'-FAM-
288 ATACAACAGCCACTTTGTGAG-3'), Caspase 2 mRNA probe (5'-Cy3-TCCAGCA
289 AGAGACTTGCACTGA-3'). Subsequently, the slips were washed with PBS three
290 times and stained with DAPI (4′, 6-diamidino- 2-phenylindole) (50 ng/mL) (Sigma,
291 USA) for 5 min, and observed by CarlZeiss LSM710 system (Carl Zeiss, Germany).

292 **The silencing or overexpression of miR-2 in mud crab**

293 The mimic of miR-2 (miR-2-mimic) or anti-miR-2 oligonucleotide (AMO-miR-2)
294 was injected into crabs at 30 μg/crab to overexpress or knockdown miR-2 in mud crab.
295 miR-2-mimic (5'-CUCACAAAGUGGCUGU**U**GU**A**U-3') and AMO-miR-2 (5'-
296 AUACAACAGCCACUUU**G**UG**A**G-3') were all synthesized by Sangon Biotech
297 (Shanghai, China) and modified with 2'-O-methyl (OME) (bold letters) and
298 phosphorothioate (the remaining nucleotides). At different time post injection, three
299 crabs from each treatment were randomly collected for later use.

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

300 **Quantification of miR-2 with qPCR**

301 Total RNA of mud crab was extracted via MagMAX mirVana Total RNA Isolation
302 Kit (Thermo Fisher Scientific, USA), then, the isolated RNAs were subjected to first-
303 strand cDNA synthesis using (5'- GTCGTATCCAGTGCAGGGTCCGAGGTCACTG
304 GATACGACATACAACA-3') by PrimeScriptTM II 1st Strand cDNA Synthesis Kit
305 (Takara, Japan). After that, Premix Ex Taq (TaKaRa, Japan) was used to quantify the
306 expression level of miR-2, U6 was used as the internal control. The relevant primers
307 used were listed below, miR-2 (5'-CGCCGCTCACAAAGTGGC-3' and 5'-
308 TGCAGGGTCCGAGGTCACTG-3'), U6 (5'-CTCGCTTCGGCAGCACA-3' and 5'-
309 AACGCTTCACGAATTTGCGT-3').

310 **Statistical analysis**

311 All the numerical data presented were analyzed by one-way analysis of variance
312 (ANOVA) to calculate the means and standard deviations of triplicate assays. The
313 differences were considered statistically significant at $P<0.05$ and $P<0.01$.

314

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

315 **Acknowledgments**

316 This study was financially supported by the National Natural Science Foundation
317 of China (31802341, 31902406), STU Scientific Research Foundation for Talents
318 (NTF18001).

319 **Author contributions**

320 YG and JC performed the experiments and analysed the data, YLC provided
321 technical supports, YG and SKL wrote the manuscript. All authors read and approved
322 the contents of the manuscript and its publication.

323 **Disclosure statement**

324 The authors declare no conflicts of interest.

325

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

326 **References**

327 1. Loker ES, Adema CM, Zhang SM, Kepler TB. Invertebrate immune systems—
328 not homogeneous, not simple, not well understood. *Immunol Rev*, 2010, 198(1):
329 10-24.
330 2. Leclerc V, Reichhart JM. The immune response of Drosophila melanogaster.
331 *Immunol Rev*, 2010, 198(1): 59-71.
332 3. Ying S, Pettengill M, Ojcius DM., Häcker G. Host-cell survival and death
333 during chlamydia infection. *Curr Immunol Rev*, 2007, 3(1): 31-40.
334 4. Brenner C, Kroemer G. Mitochondria—the death signal integrators. *Science*,
335 2000, 289(5482): 1150-1151.
336 5. Kockx MM, Knaapen MWM. The role of apoptosis in vascular disease. *J Pathol*,
337 2000, 190(3): 267-280.
338 6. Clarke P, Tyler KL. Apoptosis in animal models of virus-induced disease. *Nat*
339 *Rev Microbiol*, 2009, 7(2): 144-155.
340 7. Croft, SN, Walker EJ, Ghildyal R. Picornaviruses and apoptosis: subversion of
341 cell death. *mBio*, 2017, 8(5): e01009-17.
342 8. Fan TJ，Han LH，Cong RS，Liang J. Caspase family proteases and apoptosis.
343 *Acta Biochim Biophys Sin*, 2005, 37(11): 719-727.
344 9. Tang Y, Wells JA, Arkin MR. Structural and enzymatic insights into caspase-2
345 protein substrate recognition and catalysis. *J Biol Chem*, 2011, 286(39): 34147-
346 34154.

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

347 10. Troy CM, Ribe EM. Caspase-2: vestigial remnant or master regulator? *Sci*
348 *Signal*, 2008, 1(38): 42.
349 11. Krumschnabel G, Sohm B, Bock F, Manzl C, Villunger A. The enigma of
350 caspase-2: the laymen's view. *Cell Death Differ*, 2009, 16(2): 195-207.
351 12. Vakifahmetoglu-Norberg H, Zhivotovsky B. The unpredictable caspase-2: what
352 can it do? *Trends Cell Biol*, 2010, 20(3): 150-159.
353 13. Cao X, Bennett RL, May WS. c-Myc and caspase-2 are involved in activating
354 Bax during cytotoxic drug-induced apoptosis. *J Biol Chem*, 2008, 283(21):
355 14490-14496.
356 14. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, et al.
357 A microRNA polycistron as a potential human oncogene. *Nature*, 2005,
358 435(7043): 828-833.
359 15. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and
360 stability by microRNAs. *Annu Rev Biochem*, 2010, 79(1): 351-379.
361 16. Liu J, Dou Y, Sheng M. Inhibition of microRNA-383 has tumor suppressive
362 effect in human epithelial ovarian cancer through the action on caspase-2 gene.
363 *Biomed Pharmacother*, 2016, 83: 1286-1294.
364 17. Tao S, Zhang X, Zhang L, Dong J, Cai W, Gao J, et al. miR-708 promotes the
365 development of bladder carcinoma via direct repression of Caspase-2. *J Cancer*
366 *Res Clin Oncol*, 2013, 139(7): 1189-1198.

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

367 18. Corbel V, Zuprizal, Z Shi, C Huang, Sumartono, J-M Arcier, et al. Experimental
368 infection of European crustaceans with white spo syndrome virus (WSSV). *J*
369 *Fish Dis*, 2001, 24(7): 377-382.

370 19. Marc K. Viral infection and apoptosis. *Viruses*, 2017, 9(12): 356.

371 20. Shu L, Zhang X. Shrimp miR-12 suppresses white spot syndrome virus
372 infection by synchronously triggering antiviral phagocytosis and apoptosis
373 pathways. *Front Immunol*, 2017, 8: 855.

374 21. Galluzzi L, Brenner C, Morselli E, Touat Z, Kroemer G. Viral control of
375 mitochondrial apoptosis. *PLoS Pathog*, 2008, 4(5): e1000018.

376 22. Bitko V, Shulyayeva O, Mazumder B, Musiyenko A, Ramaswamy M, Look DC,
377 et al. Nonstructural proteins of respiratory syncytial virus suppress premature
378 apoptosis by an NF-kappaB-dependent, interferon-independent mechanism and
379 facilitate virus growth. *J Virol*, 2007, 81(4): 1786-1795.

380 23. Huang T, Cui Y, Zhang X. Involvement of viral microRNA in the regulation of
381 antiviral apoptosis in shrimp. *J Virol*, 2014, 88(5): 2544-2554.

382 24. Duan H, Dixit VM. RAIDD is a new 'death' adaptor molecule. *Nature*, 1997,
383 385(6611): 86-89.

384 25. Andersen JL, Johnson CE, Freel CD, Parrish AB, Day JL, Buchakjian MR, et
385 al. Restraint of apoptosis during mitosis through interdomain phosphorylation
386 of caspase-2. *Embo J*, 2009, 28(20): 3216-3227.

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

387 26. Terry MR, Arya R, Mukhopadhyay A, Berrett KC, Clair PM, Witt B, et al.
388 Caspase-2 impacts lung tumorigenesis and chemotherapy response in vivo. *Cell*
389 *Death Differ*, 2015, 22(5): 719-730.
390 27. Taghiyev AF, Rokhlin OW, Glover RB. Caspase-2–based regulation of the
391 androgen receptor and cell cycle in the prostate cancer cell line LNCaP. *Genes*
392 *Cancer*, 2011, 2(7): 745-752.
393 28. Umbach JL, Cullen BR. The role of RNAi and microRNAs in animal virus
394 replication and antiviral immunity. *Genes Dev*, 2009, 23(10): 1151-1164.
395 29. Murchison EP, Hannon GJ. miRNAs on the move: miRNA biogenesis and the
396 RNAi machinery. *Curr Opin Cell Biol*, 2004, 16(3): 223-229.
397 30. Yang L, Yang G, Zhang X. The miR-100-mediated pathway regulates apoptosis
398 against virus infection in shrimp. *Fish Shellfish Immunol*, 2014, 40(1): 146-153.
399 31. Magenta A, Cencioni C, Fasanaro P, Zaccagnini G, Greco S, Sarra-Ferraris G,
400 et al. miR-200c is upregulated by oxidative stress and induces endothelial cell
401 apoptosis and senescence via ZEB1 inhibition. *Cell Death Differ*, 2011, 18(10):
402 1628-1639.
403 32. Chen J, Gong Y, Zheng H, Ma H, Aweya JJ, Zhang Y, et al. *Sp*Bcl2 promotes
404 WSSV infection by suppressing apoptotic activity of hemocytes in mud crab,
405 *Scylla paramamosain*. *Dev Comp Immunol*, 2019, 100: 103421.
406 33. Hu H, Zhao X, Gong Y. *Sp*ICAD contributes to WSSV infection by suppressing
407 apoptosis in mud crab (*Scylla paramamosain*). *Aquaculture*, 2021, 536: 736449.
408

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

409 **Figure legends**

410 **Fig 1. Bioinformatics analysis of *Sp*-Caspase 2. (A)** Schematic view of the structure
411 of Caspase 2 protein. **(B)** The three-dimensional model of Caspase 2 protein. **(C)**
412 Multiple alignments of the deduced amino acid sequence of Caspase 2 protein in mud
413 crab and other marine crustaceans. Conserved active sites (H158, C200) were marked
414 with the red box, proteolytic cleavage sites (S207, P230) were marked with the green
415 box. Proteins analyzed listed below: Pt-Caspase 2 (ARO92228.1) from *Portunus.*
416 *trituberculatus*, Es-Caspase 2 (AGT29867.1) from *Eriocheir sinensis*, Lv-Caspase 2
417 (AGL61581.1) from *Litopenaeus vannamei*, Pm-Caspase 2 (ABO38430.1) from
418 *Penaeus monodon* and Fc-Caspase 2 from *Fenneropenaeus chinensis* (ALL27850.1).

419 **Fig 2. Role of Caspase 2 on virus infection in mud crab. (A)** mRNA levels of Caspase
420 2 during virus infection in mud crabs. β-actin was used as an internal control. **(B)**
421 Protein levels of Caspase 2 in the hemocytes of WSSV challenged crabs. Tubulin was
422 used as an internal control. **(C)** The efficiency of Caspase 2 silence. Mud crabs were
423 treated with Caspase 2-siRNA or Caspase 2-siRNA-scrambled, at 48 h post-injection,
424 the Caspase 2 protein of hemocytes was detected by western blot. **(D)** The influence of
425 Caspase 2 knockdown on WSSV infection in mud crab. Mud crabs were co-injected
426 WSSV and Caspase 2-siRNA, followed by the expression detection of viral gene VP28
427 by qPCR. All data were the average from at least three independent experiments, mean
428 ± s.d. (**, $p<0.01$).

429 **Fig 3. The function of Caspase 2 in regulating apoptosis. (A-B)** The effect of Caspase
430 2 on apoptosis regulation of mud crab hemocytes. Mud crabs were injected with WSSV

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

431 or co-injected with WSSV and Caspase 2-siRNA, then. the apoptotic levels of mud crab
432 hemocytes were examined through the Caspase 3/7 activity detection **(A)** and annexin
433 V analysis **(B)**. Data was shown as mean values ± standard deviations. Asterisks
434 indicated significant differences (*P<0.05 and **P<0.01).

435 **Fig 4. The interactions between miR-2 and Caspase 2 in mud crab. (A)** The
436 prediction of miRNA that targeting Caspase 2. Target Scan, RNAhybrid and miRanda
437 algorithms were used for the prediction analysis, as predicted, miR-2 could target the
438 3'UTR of Caspase 2. **(B)** The construction of the plasmid EGFP-Caspase 2 3'UTR or
439 EGFP-Δ Caspase 2 3'UTR. The seed sequence targeted by miR-2 was underlined. **(C)**
440 The interaction between miR-2 and Caspase 2 3'UTR in S2 cells. S2 cells were co-
441 transfected with miR-2 and the indicated constructed plasmids (EGFP- Caspase 2
442 3'UTR or EGFP-Δ Caspase 2 3'UTR), then the fluorescence intensity of S2 cells was
443 detected and analyzed. **(D)** The co-localization of Caspase 2 mRNA and miR-2 in mud
444 crab hemocytes. Caspase 2 mRNA probe was labeled with Cy3 (red), miR-2 probe was
445 labeled with FAM (green), Scale bar, 10 μm. Significant statistical differences between
446 treatment were indicated with asterisks (**, $p<0.01$).

447 **Fig 5. The effects of miR-2 on Caspase 2 expression in mud crab. (A)** The influence
448 of miR-2 silencing on the expression level of Caspase 2 in mud crabs injected with
449 either AMO-miR-2 or AMO-miR-2-scrambled, and the mRNA and protein levels were
450 detected at 48 h post-injection. **(B)** The influence of miR-2 overexpression on the
451 expression level of Caspase 2 in mud crabs injected with either miR-2 or miR-2-
452 scrambled, and the mRNA and protein levels were detected at 48 h post-injection. Each

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

453 experiment was performed in triplicate and data are presented as mean ± s.d. (**,
454 p<0.01).

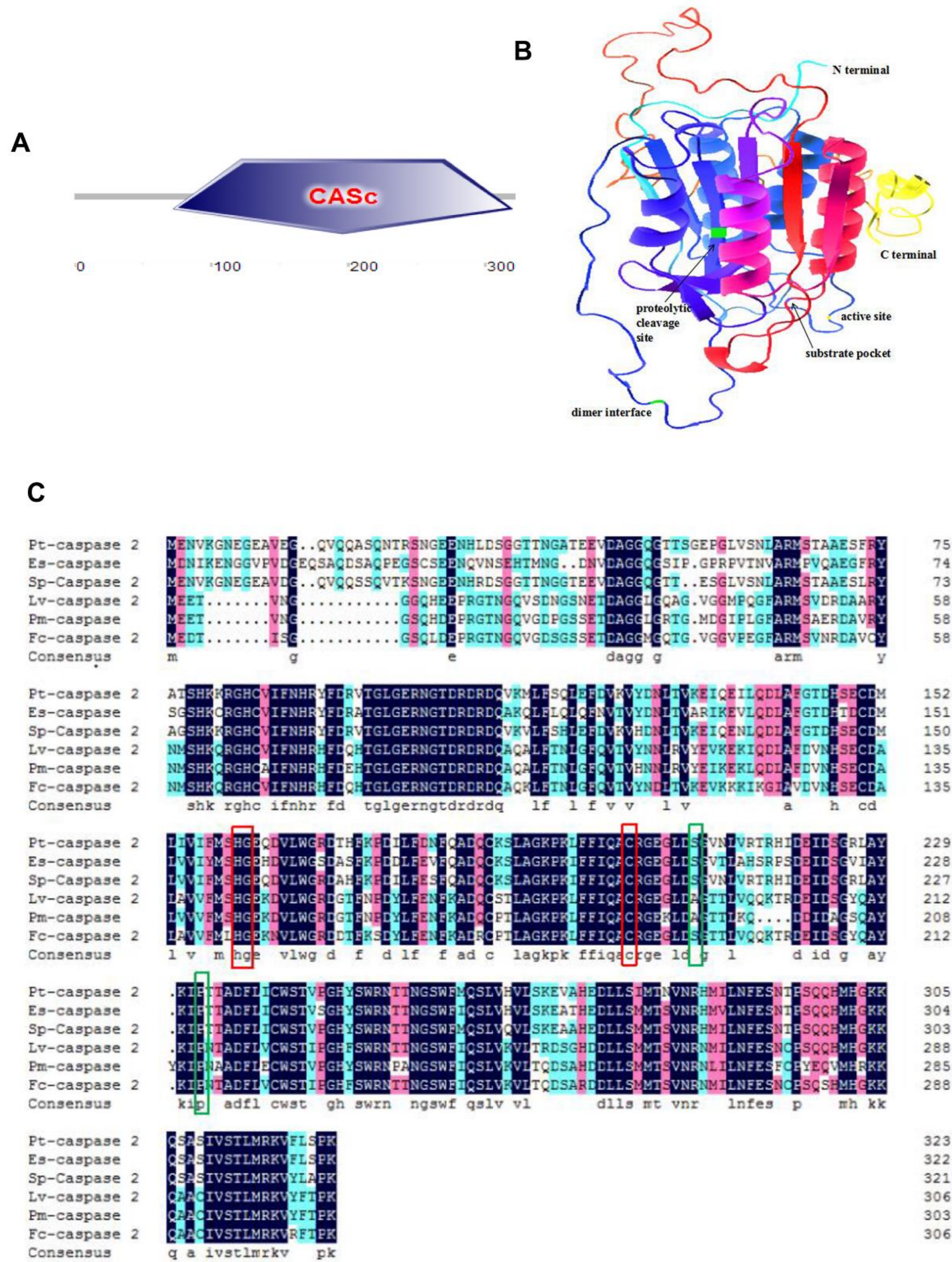
455 **Fig 6. miR-2 promotes WSSV proliferation by targeting Caspase 2 in mud crab.**
456 **(A)** The detection of miR-2 expression during WSSV infection in mud crab using qPCR
457 analysis. **(B)** The expression of virus gene VP28 in mud crabs co-treated with WSSV
458 and AMO-miR-2, AMO-miR-2-scrambled was used as control. **(C)** The effects of miR-
459 2 overexpression on the expression of virus gene VP28 in mud crab during WSSV
460 infection. **(D)** The involvement of Caspase 2 during miR-2 -mediated virus promotion,
461 AMO-miR-2, WSSV and Caspase 2-siRNA were co-injected into mud crabs, followed
462 by the detection of WSSV copy numbers. All the numeral data represented the mean ±
463 s.d. of triplicate assays (**, p<0.01).

464 **Fig 7. miR-2 suppresses apoptosis by targeting Caspase 2 in mud crab. (A-B)** The
465 involvement of Caspase 2 during the miR-2-mediated apoptosis regulation. Mud crabs
466 were treated with either AMO-miR-2 or co-treated with AMO-miR-2 and Caspase 2-
467 siRNA, followed by apoptosis evaluation in mud crab hemocytes through the Caspase
468 3/7 activity analysis **(A)** and annexin V assay **(B)**. Data presented were representatives
469 of three independent experiments (**, *p*<0.01).

470 **Fig 8. Proposed schematic diagram for miR-2-Caspase 2 pathway in regulating**
471 **apoptosis and virus infection in mud crab.**

472

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



473

474

475

476

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Fig. 2

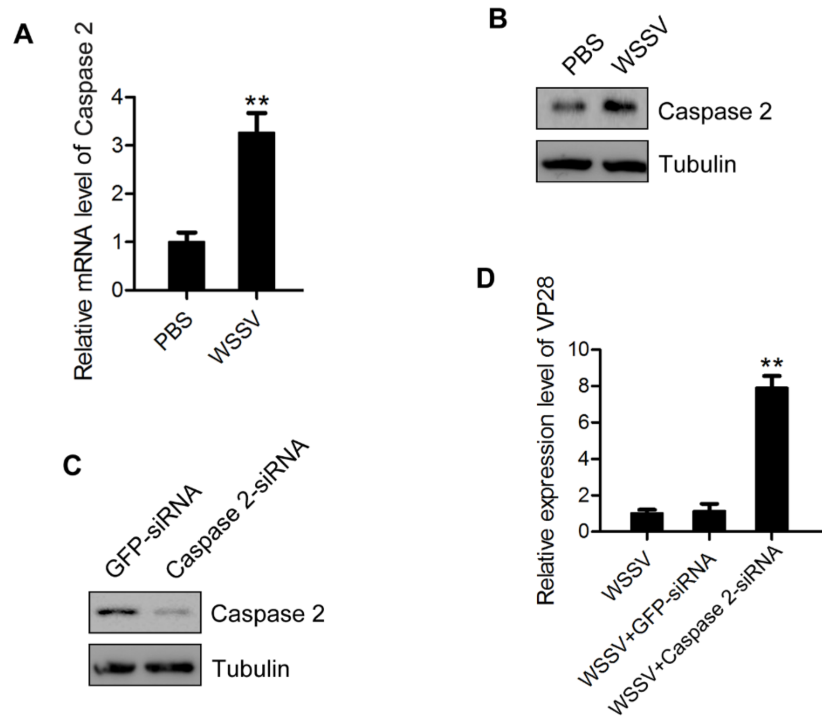
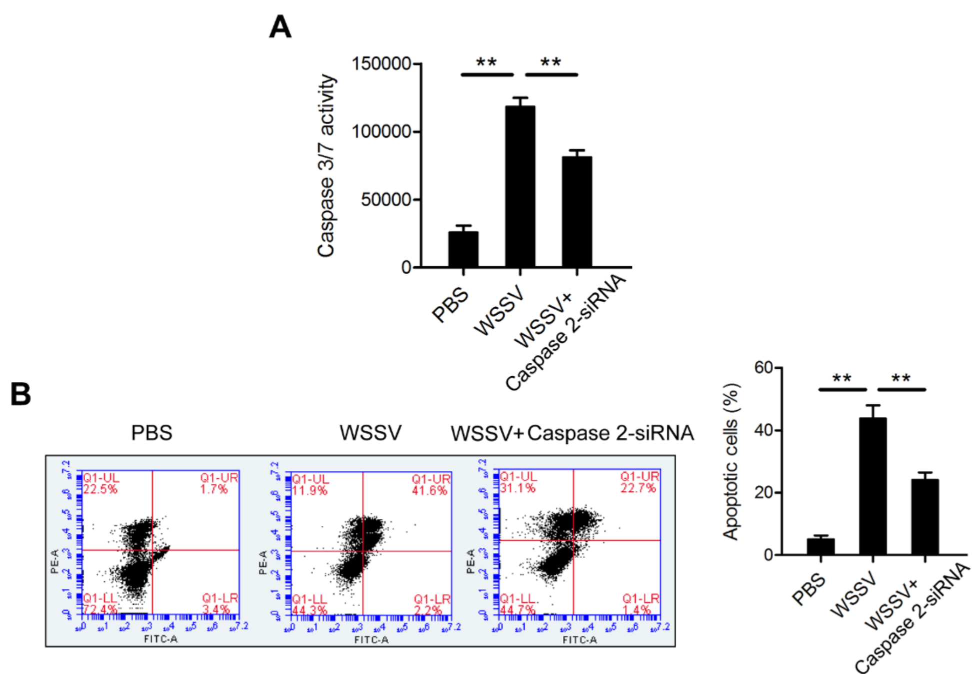
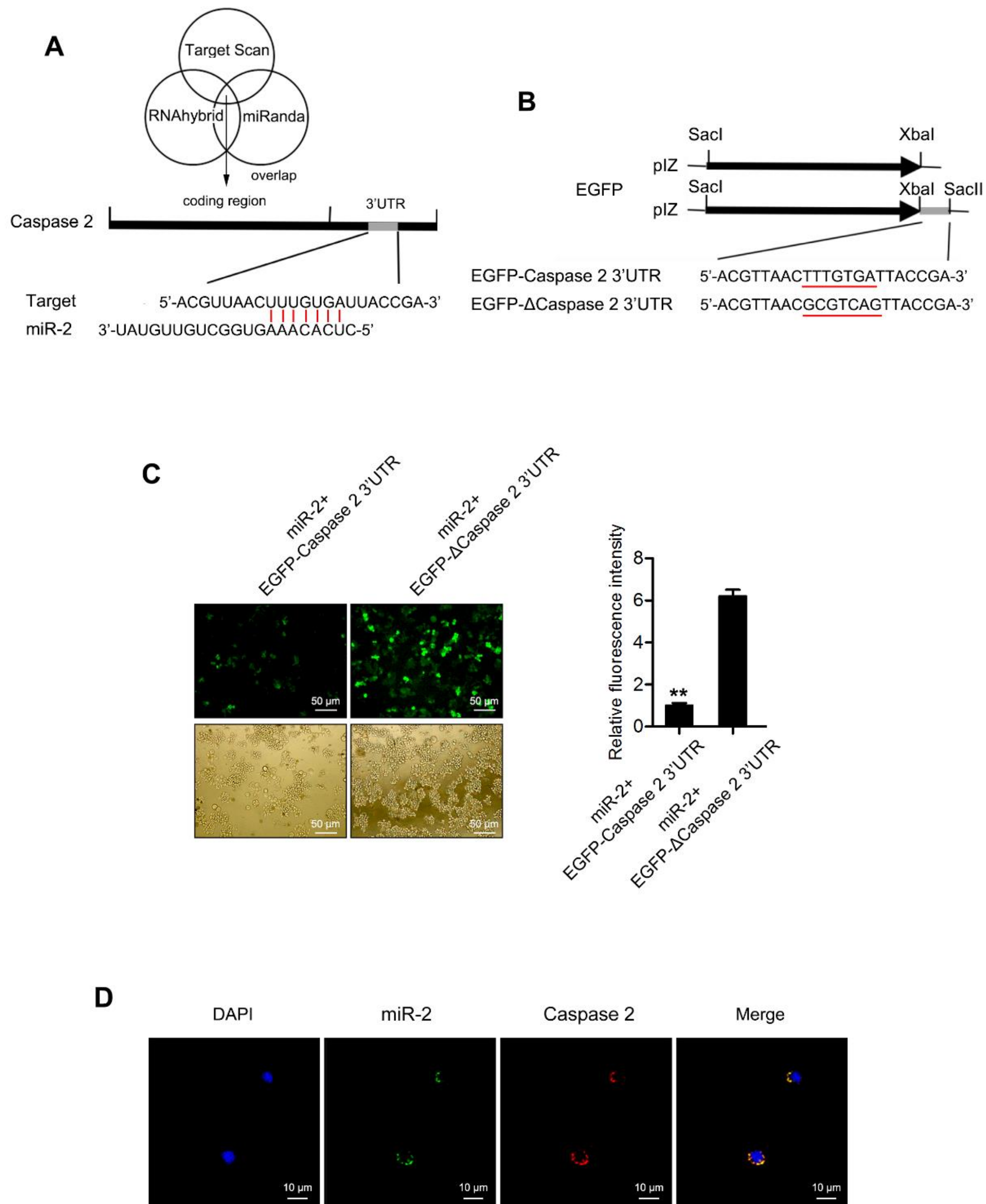


Fig. 3



bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



478

479

480

481

482

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Fig. 5

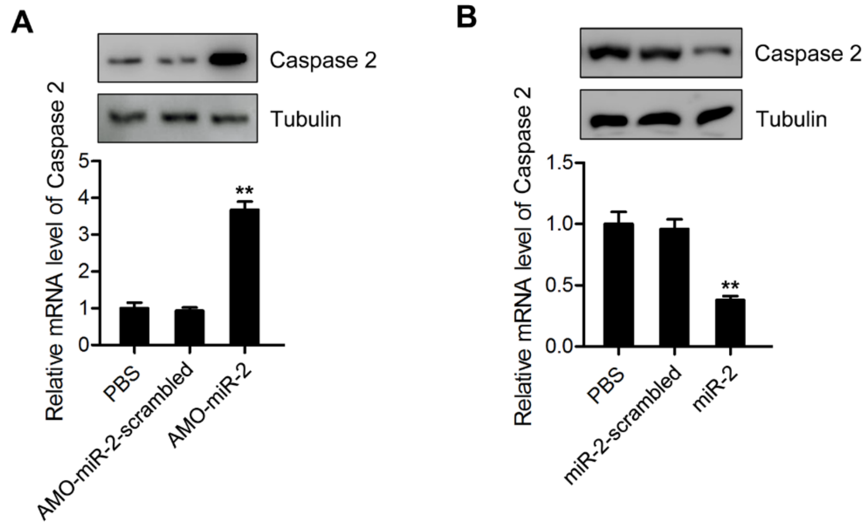
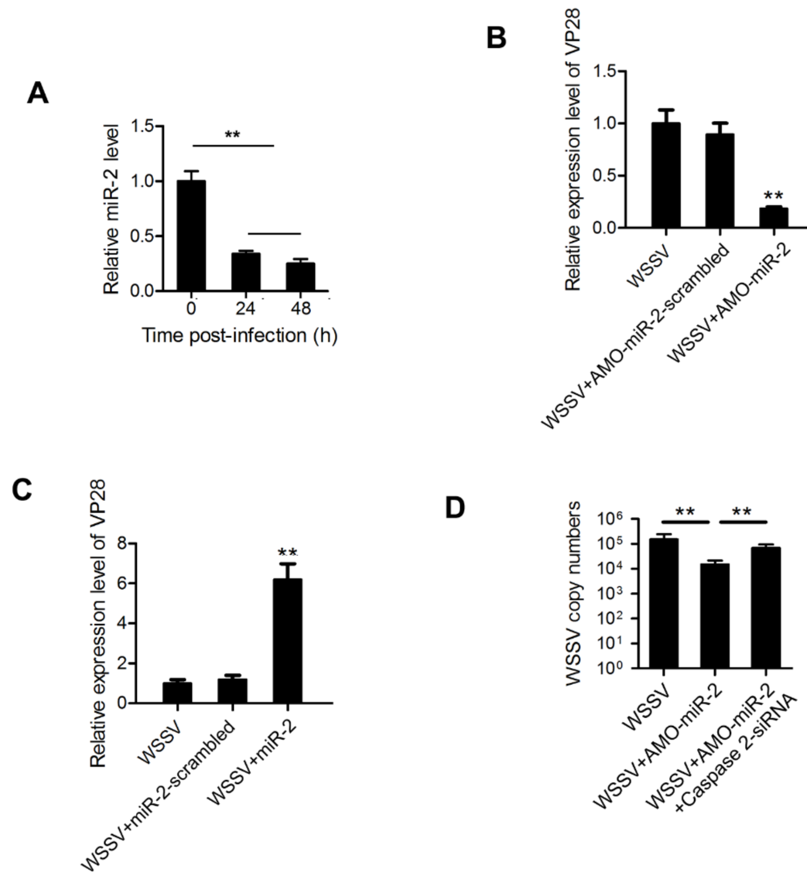


Fig. 6



bioRxiv preprint doi: https://doi.org/10.1101/2021.05.17.444593; this version posted May 18, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Fig. 7

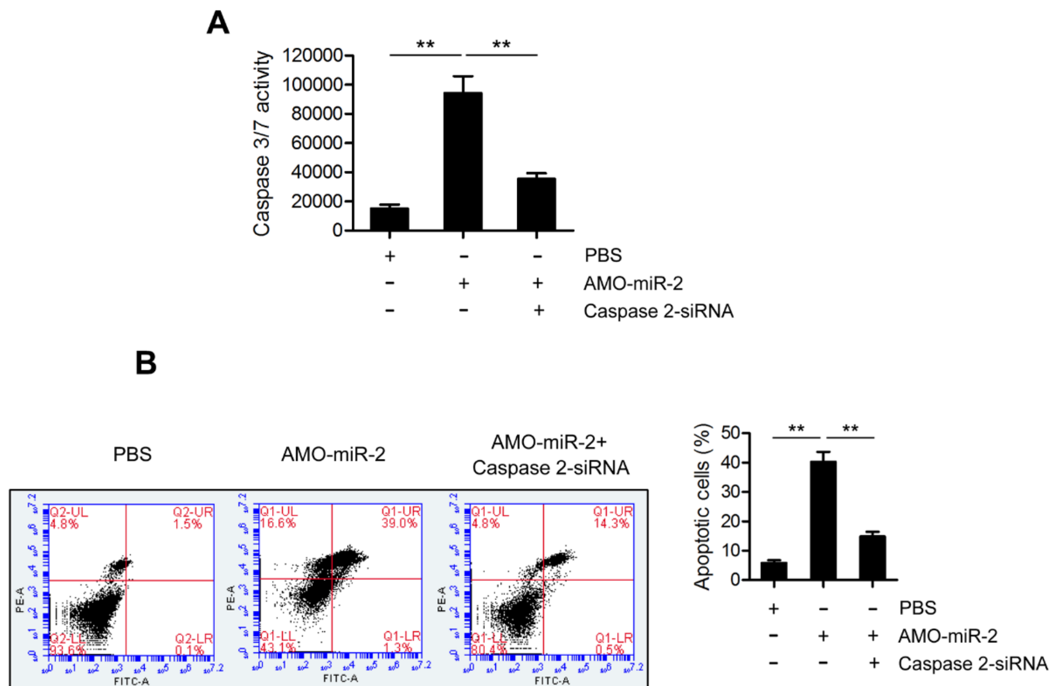


Fig. 8