

1 **Degradation of miR-466d-3p by JEV NS3 facilitates viral replication and IL-1 β**
2 **expression**

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4 **Short title: miRNA degradation by NS3 of JEV**

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20 **Author's contributions**

21 YY and GPL conceived the study, HJ, CQZ, ZFB, YQM, TQ and JY performed all
22 experiments, YY wrote the manuscript, and SBC, GJW and GPL edited, all of the authors
23 critically reviewed and approved the final manuscript.

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25

26 **Abstract**

27 Previous studies revealed that Japanese encephalitis virus (JEV) infection alters the
28 expression of miRNA in central nervous system (CNS). However, the mechanism of JEV
29 infection contributes to the regulation of miRNAs in CNS remain obscure. Here, we found
30 that a global degradation of mature miRNA in mouse brain and neuroblastoma cells after JEV
31 infection. In additional, the integrative analysis of miRNAs and mRNAs suggests that those
32 down-regulated miRNAs are primarily targeted inflammation genes and the miR-466d-3p
33 target the IL-1 β which in the middle of those inflammation genes. Transfection of
34 miR-466d-3p decreased the IL-1 β expression and inhibited the JEV replication in NA cells.
35 Interestingly, the miR-466d-3p level increased after JEV infection in the presence of
36 cycloheximide, which indicated that viral protein expression reduces miR-466d-3p.
37 Therefore, we generated all the JEV coding protein and demonstrated that NS3 is a potent
38 miRNA suppressor. Furthermore, the NS3 of ZIKA virus, WNV, DENV1 and DENV2 also
39 decreased the expression of miR-466d-3p. The in vitro unwinding assay demonstrated that the
40 NS3 could unwind the pre-miR-466d and induce the dysfunction of miRNA. Using
41 computational models and RNA immunoprecipitation assay, we found that arginine-rich
42 domains of NS3 are critical for pre-miRNA binding and the degradation of host miRNAs.
43 Importantly, site-directed mutagenesis of conserved residues revealed that R226G and
44 R202W significantly reduced the binding affinity and degradation of pre-miR-466d.
45 Together, these results extend the helicase of Flavivirus function beyond unwinding duplex
46 RNA to the decay of pre-miRNAs, which provides a new mechanism of NS3 in regulating
47 miRNA pathways and promoting the neuroinflammation.

48

49 **Author Summary**

50 Host miRNAs had been reported to regulate JEV induced inflammation in central nervous
51 system. We found that the NS3 of JEV can reduce most of host miRNA expression. The
52 helicase region of the NS3 specifically binds to precursors of miRNA and lead to incorrect
53 unwinding of precursors of miRNAs which inhibits the function of miRNAs. This observation
54 leads to two major findings. First, we identified the miR-466d-3p targets to the host IL-1 β and
55 E protein of JEV, and NS3 degrades the miR-466d-3p to promote the brain inflammation and
56 viral replication. Second, we proved that the arginine on the helicase of NS3 is the main
57 miRNA binding sites, and the miRNA degradation by NS3 was abolished when the R226 and
58 R202 were mutated on the NS3. These findings were also confirmed with NS3 of ZIKA virus,
59 WNV and DENV which could decrease the expression level of miR-466d-3p to enhance the
60 inflammation. Our study provides new insights into the molecular mechanism of encephalitis
61 caused by JEV, and reveals several amino acid sites to further attenuate the JEV vaccine.

62

63

64 **Introduction**

65 JEV is a single-stranded, positive-sense RNA virus belonging to flavivirus of the Flaviviridae
66 family. Its genome encodes three structural proteins in order of envelope (E), capsid (C), and
67 premembrane (PrM), and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A,
68 NS4B, and NS5. During the JEV life cycle in cells, the replication was initiated with the RNA
69 genome replicase complex, of which NS3 and NS5 are major components required for
70 genome replication and capping (11). The polyprotein NS3 of JEV belongs to superfamily 2
71 helicase, which has protease, nucleoside triphosphatase, ATP-dependent RNA helicase
72 activities and unwind double strands genome during the viral replication (63).

73 JEV is a neurotropic virus causing neuroinflammation and neuronal damage that causes
74 Japanese encephalitis (JE) (44). In spite of vaccines available control JEV, approximately
75 67,900 cases of JE are reported around the world per year, half of which were occurred in the
76 mainland of China (7). Due to the lack of effective anti-viral drug against JEV, the fatality
77 rate of JE is ~25-32%, and 50% of the survivors suffer from neuropsychiatric sequelae (55).
78 JEV causes neuroinflammation and neuronal damage in mammalian hosts by modulating
79 cytokine/chemokine production (10) as well as the activation and migration of neuroglial cells
80 (12, 19). The production of inflammation cytokine increases microglial activation following
81 JEV infection, which facilitates outcome of viral pathogenesis and benefit the dissemination
82 of the virus in the CNS (25, 35). For this reason, most of studies on innate immune response
83 in the CNS have focused on the molecular components of JEV recognition and signaling
84 regulator. Recent studies have revealed that the JEV was recognized by the toll-like receptor 7
85 (TLR7) in neuron cells and TLR3 in microglia cells (28, 47). Following JEV recognition, the
86 adapter proteins of TLRs are activated and involved myeloid differentiation primary response
87 88 (MyD88). Furthermore, protein kinases such as kinase B (Akt), phosphoinositide 3-kinase
88 (PI3K), p38 mitogen-activated protein kinase (p38MAPK) and signal-regulated kinase (ERK)
89 (18) are triggered by JEV infection. However, the involvement of JEV components in
90 modulating inflammation response still obscure. To our knowledge, several recent studies
91 found that ZIKV NS5 facilitates inflammasome to induce IL-1 β secretion (66). JEV NS3
92 interacts with Src protein tyrosine kinase promote inflammatory cytokine secretion (50).

93 DENV NS1 has been reported to interact with STAT3 that enhance the secretion of TNF- α
94 and IL-6 (18). Hence, the non-structural proteins of Flaviviridae play an important role in
95 inflammation response, but the specific signaling pathway and target sites of JEV
96 non-structural protein remains unknown.

97 Regardless of species of origin, microRNAs (miRNA) are small, noncoding RNAs containing
98 ~22 nucleotides (nt) that control the posttranscriptional gene regulation by targeting mRNAs.

99 In animal cell, following transcription by RNA polymerase II (RNA Pol II), the RNA
100 polymerase III (RNA Pol III) Droscha in the nucleus process the primary transcripts of

101 miRNAs (pri-miRNAs) into ~60-100 nt precursors (pre-miRNAs). The pre-miRNAs are then
102 shuttled into cytoplasm, and further processed by RNA Pol II Dicer into ~22 nt

103 double-stranded (ds) RNA product containing the mature miRNA guide strand and the
104 passenger miRNA strand. The mature miRNA is then load into the RNA-induced silencing

105 complex (RISC) and bind 3' untranslated regions (UTRs) of target mRNA, which lead to
106 translation repression and/or mRNA degradation. Through the repression of target, miRNA

107 modulate a broad range of gene expression programs during development, immunoreaction
108 and in pathogen infection. Accumulating evidences also suggest abundant of cellular miRNAs

109 involved in multiple stages of the JEV replication cycle. For instance, miR-155, miR-15b
110 miR-19b and miR-29b are induced and activated innated immune response after JEV

111 infection, respectively (3, 60, 61, 73). Beside JEV infection reduces the expression of
112 miR-33a and miR-432 to facilitate virus replication (14, 53). Therefore, the regulation of

113 miRNA is important for cell biological process and mRNA homeostasis during the JEV
114 infection. In contrast to the biosynthetic of miRNA, under physiological or pathological

115 conditions, some of the miRNA decayed rapidly. Most studies on degradation of miRNA
116 have demonstrated the mechanisms involved sequence organization, ribonuclease activity,

117 transcription rates and energy metabolism. For example, several 3' to 5' or 5' to 3'
118 miRNA-degrading ribonuclease were found to perform degradation of different miRNA in

119 variety of cell types (9, 30). A sequence target-dependent mechanism has also been identified,
120 in which cleavage is mediated by miRNA and high complementary interaction, such as viral

121 non-coding transcript (8, 41), miRNA and miRNA hybrid (13) or extent of sequence
122 complementarity (2). Although the miRNA function has been well defined during the viral

123 replication, the turnover dynamics of miRNA and the mechanisms involved have been poorly
124 defined.

125 Numerous studies of virus in regulating host miRNA to control cellular protein expression,
126 suggesting a critical role of host miRNAs in immune evasion and viral replication cycle. Both
127 DNA and RNA viruses have developed several mechanisms to degrade or promote cellular
128 miRNA to benefit viral infection and replication. Specifically, herpesvirus saimiri (HVS) and
129 murine cytomegalovirus (MCMV) directly degrade host miR-27 using a virus encode ncRNA
130 which contains miR-27 sequence-specific binding site (8, 39). Similarly, human
131 cytomegalovirus (HCMV) decrease the mature miR-17 and miR-20a using a 15 kb
132 microRNA (miRNA) decay element in the UL/b' region of the viral genome (37). On the
133 other hand, vaccine virus (VACV) degrades the host miRNA via adding tails using viral
134 poly(A) polymerase (4). Furthermore, the Kaposi's sarcoma-associated herpesvirus (KSHV)
135 repress the MCP1 to facilitate its own miRNA expression (29). Although the direct
136 interaction between host miRNAs and viral components remain far behind other studies, to
137 date, there are two approaches to predict RNA and protein interaction. One of the most
138 cost-effective approaches is computational predicting methods, which could predict
139 protein-RNA interaction and protein-RNA binding sites. Given the limited accurate of
140 computational methods, the RNA-protein immunoprecipitation provides a specific and
141 accurate predication of RNA-protein interaction. Based on those approaches, those would be
142 especially valuable to mapped miRNA-protein binding sites and characterized biological
143 function of viral components in viral replication process.

144 In the present study, JEV NS3 has been demonstrated to directly disrupt cellular pre-miRNA
145 and reduce mature miRNA levels. Through computational and RNA immunoprecipitation
146 (RIP) analysis, the miRNA binding and turnover of NS3 was found to be arginine-rich
147 domains dependent, which was determined by R226G and R202W of NS3. In addition, NS3
148 mediated miRNA degradation is critical in inflammation related pathway, which may
149 promote an irreversible inflammatory response leading to neuronal cell death. These results
150 also provide further insight into the role of flavivirus helicase in the regulation of host
151 miRNA turnover.

152 **Results**

153 **JEV mediates down regulation of global miRNA in mice nervous system**

154 To examine the host miRNAs expression profiles that regulated by JEV infection, miRNA
155 microarrays were used to assay host miRNA expression profiles in mice brain with infection
156 of pathogenic strain JEV (P3). The miRNA expression profiles showed that the abundance of
157 host miRNAs was altered during JEV infection (Fig. 1A). Notably, the 76.7% of total 41
158 miRNAs (p-value <0.05 and FC > 2.0) were reduced during JEV infection in mice brain. To
159 determine whether the global down regulation of endogenous miRNAs was biased in any
160 way, we used a small RNA deep sequence to analysis the miRNA expression profile of P3
161 infected NA cells at MOI of 1 and 0.1 for 48 hours (Fig. 1B). This analysis further confirmed
162 that JEV infection led to global host miRNAs decrease. Furthermore, the virus titer and NS3
163 expression level of cells infected with P3 at 1 MOI was higher than cell infected with P3 at
164 0.1 MOI, and 1 MOI infection more significantly decreased miRNA expression than 0.1 MOI
165 infection, suggesting that the JEV induced the global miRNA decrease is related to JEV
166 replication (Fig. 1C and D). The miRNA profiles were also confirmed by qRT-PCR analyses
167 in JEV infected NA, BV2 and bEnd.3 cells (Fig. 1E). The integrative analysis of miRNAs and
168 mRNAs was used to predict the major target of the decreasing miRNAs. A total of 42
169 interacting proteins with 177 interactions were retrieved from the STRING database and 8
170 proteins were segregated to one subgroup, of which were related to immune and inflammation
171 process (S1 Fig). The result indicated that IL-1 β is in the middle of these influential genes and
172 the miR-466d-3p is the only miRNA that decreased significantly (FC \geq 2.0, p value < 0.01)
173 which target the IL-1 β .

174

175 **JEV infection induces incorrect processing of host miRNAs**

176 Interestingly, the mature miRNA sequence-specific reads were determined by deep
177 sequencing of the 18-24 nt fraction and analyzed by the miRDeep2 module (24), which
178 revealed that percentage of incorrect sequences in the total reads of miR-466d-3p,
179 miR-381-3p and miR-466a-3p were increased (S2 Fig). Furthermore, we observed some of
180 miRNAs from the same precursor were all down-regulated, such as, the miR-466h-3p and

181 miR-466d-3p generated from miR-466d-3p precursor (Fig. 2A), which were both
182 significantly down-regulated in NA cells (Fig. 2B). Notably, the pre-mir-466d-3p and mature
183 miR-466d-3p were reduced in a dose dependent, but the pri-miR-466d-3p appeared
184 unmodified (Fig. 2B). To further examine the miRNA degradation inducing by JEV, the JEV
185 significantly decrease the abundance of miR-466d-3p that derived from an exogenously
186 constructed miRNA mimic compared to the medium control in NA cells (Fig. 2C). Therefore,
187 these experiments indicated that JEV infection decreases the level of pre-miRNA to
188 downregulate miR-466d-3p expression.

189

190 **JEV NS3 mediates degradation of miR-466d-3p in neuronal cells**

191 Expression of the non-structure protein or noncoding sub genomic RNA (sfRNA) of virus is
192 required for viral pathogenicity which would affect the host miRNAs processing (34). JEV
193 infection of neuronal cells results in viral gene transcription and subsequently followed by
194 viral protein translation. The transcriptional inhibitor Favipiravir (T-705) and α -Amanitin or
195 the translation inhibitor cycloheximide (CHX) were used to identify the viral components
196 induced degradation of miR-466d-3p. All early treatments of T-705 and CHX at 12 hours post
197 infection (hpi) could severely block the JEV replication and inhibit degradation of the
198 miR-466d-3p in NA cells. In contrast, late treatment of CHX at 42 hpi could block the
199 degradation of miR-466, but not the treatment of T-705 at 42 hpi (Fig. 3A). Taken together,
200 these data suggested that the posttranscriptional modification or improper processing of host
201 miRNAs is dependent on viral protein production or viral protein with host miRNA
202 interaction in mice neuron cells.

203 Since JEV transcript did not suppress host miRNA, to further determine which viral protein
204 were sufficient to confer the host miRNA degradation, 8 pcDNA3.1 vector encoding each
205 structure protein (E, C and PrM) non-structure protein (NS1, NS2, NS3, NS4A, NS4B and
206 NS5) of JEV were constructed. The miR-466d-3p expression was measured 48 hours post
207 transfection. Only NS3 induced a significant decrease of mature miR-466d-3p (40% reduction
208 compare to pcDNA 3.1 control) (Fig. 3B). Several of other non-structure proteins of JEV
209 have been revolved to associate with NS3 to facilitate the RNA assembling and viral
210 replication (40, 46). To determine the potential association between other different

211 non-structure proteins of JEV, the co-transfection of NS2B, NS4A and NS5 with NS3 were
212 determined and there was no significantly different comparing to NS3 individual transfection
213 group. Moreover, the expression of NS3 in the NA cells also induced a global down
214 regulation of endogenous miRNAs (Fig. 3C) and the same miRNAs in the Fig 1E were also
215 confirmed by qRT-PCR (Fig. 3D). We further assessed the effect of JEV and NS3 in the
216 human neuroblastoma cells (SK-N-SH) to test the regulation of human miRNA by qRT-PCR.
217 As shown in Fig. 3E, both the JEV infection and the NS3 transfection reduced the miRNA
218 expression level in SK-N-SH cells.

219 Since the JEV NS3 represented a critical role in turnover of host miRNA, we constructed
220 pcDNA3.1 vector encoding NS3 plasmids of ZIKA virus, WNV, DENV1, DENV2, CSFV,
221 BVDV and HCV to identify whether the helicase of other flavivirus could degrade the host
222 miRNAs. The NS3 of ZIKA virus, WNV, DENV1 and DENV2 induced the decrement of
223 miR-466d-3p when transfected into NA cells (Fig. 3F). However, transfection with NS3 of
224 CSFV, BVDV and HCV were not affected the amount of miR-466d-3p. The alignment of
225 NS3 was revealed that JEV, ZIKA virus, WNV, DENV1 and DENV2 were included in one
226 subgroup and different from the others (CSFV, BVDV and HCV) (S3 Fig). These results
227 indicated that the NS3 of Flavivirus decrease the expression level of miR-466d-3p.

228 The hypothesis of miRNA degradation and their mRNA-targeting activities upon NS3
229 expression is not due to inhibiting the expression of RISC. This was partially confirmed by
230 qRT-PCR of the major RISC components gene expression, which of them were all
231 significantly increased after JEV infection (S4 Fig). Furthermore, to explore the possibility
232 that NS3 interacted with dicer or RISC, the proteins that co-immunoprecipitation with NS3
233 were analysis with LC-MS (S1 Table). The amino sequence identification showed 209
234 proteins which score were higher than 90 and rich in several functional categories such as,
235 heat shock protein, cytoskeletal components, chaperonin protein and TRiC (TCP-1 Ring
236 Complex). To our knowledge, none of these proteins were correlated with miRNA
237 degradation process that has been reported previously. Furthermore, transfection with NS3 in
238 NA cells significantly decrease the amount of miR-466d that derived from an exogenously
239 constructed miRNA mimic (Fig. 3G), indicating NS3 affect the processing of pre-miRNA or
240 double-stranded segment of miRNA that is 22 bp.

241

242 **Block miR-466d enhance IL-1 β secretion and promote JEV replication**

243 To verify *in silico* analysis of miRNA-mRNA interaction, the level of IL-1 β were further
244 determined in JEV-infected mice brain and cells using ELISA. The results revealed that the
245 IL-1 β level in mice brain was significantly enhanced at 6 dpi (Fig. 4A). In addition, protein of
246 IL-1 β were significantly elevated in the dose-dependent and time-dependent manner in NA,
247 BV2 and bEnd.3 cells.1 (Fig. 4B).

248 The sequences of miR-466d-3p and its target site in the 3' UTR of IL-1 β were aligned using
249 TargetScan (http://www.targetscan.org/mmu_72/) (Fig. 4C). To determine whether IL-1 β
250 mRNA is indeed target for miR-466d-3p, the expression of IL-1 β was examined in BV2 and
251 NA cells after transfected with miR-466d-3p mimics or miR-466d-3p inhibitors, respectively.
252 The results shown that overexpression of miR-466d-3p significantly suppressed IL-1 β mRNA
253 and protein levels in BV2 and NA cells, and the similar results were observed in BV2 and NA
254 cells after infected with JEV or treated with LPS (Fig. 4D-F, top). In contrast, miR-466d-3p
255 inhibitor was significantly increased IL-1 β mRNA expression and protein secretion in BV2
256 and NA cells, respectively (Fig. 4D-F, bottom). Moreover, the miR-466d-3p expression level
257 of the cells treated with LPS or poly(I:C) was similar to the medium treated cells and
258 significantly higher than JEV infected cells. However, the miR-466d-3p mimic could still
259 inhibit the LPS or poly(I:C) induced IL-1 β over-transcription and overexpression, which
260 indicated that miR-466d-3p is a common regulator of IL-1 β not specific to the JEV infection.

261 Interestingly, a miRNA and JEV viral genomic gene comparison analysis indicated that the
262 miR-466d-3p also targets the coding sequence of JEV E, NS3 and NS5 genomes (Fig. 4C).
263 There was a substantial reduction of virus titer in the NA cells that were transfected with
264 miR-466d-3p. Moreover, inhibition of miR-466d-3p leads to an increment of JEV replication
265 (Fig. 4G). To determine whether the miR-466d-3p direct target the seed sequence of JEV, we
266 constructed a miR-466d target sites-fused GFP expressing vector which containing 2
267 miR-466d target seed sequence of JEV NS3 and E. As expected, transfection of miR-466d
268 mimic resulted in a complete loss fluorescence in miR-466d target sites-fused GFP expression
269 cells, whereas co-transfection of NS3 significant recovered expression of GFP, suggesting
270 that NS3 cleave miR-466d to block the miRNA function (Fig. 4H). Thus, these data suggested

271 IL-1 β as a possible target of miR-466d-3p which also worked as a negative regulator of JEV
272 replication.

273

274 **Unwinding of miR-466d-3p by NS3 blocks the silencing function of miRNA**

275 The NS3 of JEV previously has been identified as an RNA helicase that acts by unwinding of
276 dsRNA (63). The transactivating response RNA-binding protein (TRBP) and Argonaute 2
277 contain several dsRNA-binding domains, which facilitate the dsRNA fragments into RISC in
278 order to target mRNA (15, 64). We hypothesized that host pre-miRNAs may also be unwound
279 into a single strand RNA by NS3, in which the RISC could not recognize the single strand
280 fragment or target the mRNA. In vitro unwinding assays revealed that a synthesized
281 pre-miR-466d-3p containing a hairpin structure (Fig. 5A, top) was unwound into a single
282 strand RNA in a dose dependent manner (from lane 2 to lane 5 of Fig 5B). To determine
283 whether NS3 can directly unwind miR-466d-3p mimic in addition to endogenous host
284 miRNAs, using in vitro degradation assay, the synthetic double strand miR-466d-3p mimic
285 (Fig. 5A, bottom) could also unwind into a single strand by NS3 in a dose dependent manner
286 (from lane 2 to lane 5 of Fig. 5C).

287 To further examine whether the unwinding miRNA could still transport into RISC and
288 execute a normal function of RNA silencing with host mRNA. The fragment of unwinding
289 miR-466d-3p mimic and pre-miR-466d-3p were recycled with Trizol after the in vitro
290 unwinding assay. After transfection with unwinding miR-466d-3p mimic, the expression level
291 of mature miR-466d-3p was similar to the NC control group, indicating that the unwinding
292 miR-466d-3p mimic was unstable in the host cells (Fig. 5D). Notably, we also did not observe
293 the mRNA expression and protein secretion level of IL-1 β decreased after transfection of
294 unwinding miR-466d-3p mimic with or without JEV infection (Fig. 5E and 5F). Taken
295 together, these data demonstrate that the NS3 of JEV could facilitate the disfunction of
296 miR-466d-3p in the host cells.

297

298 **NS3 has specific binding affinity with pre-miRNA**

299 Many VSRs have been reported to block RNAi by binding siRNA or miRNAs (27, 52).
300 Several ribonucleoproteins performed the post-transcriptional regulatory networks that are

301 mediated by RNA-protein interactions (RPIs), and some computational models were
302 developed to help identify the RPIs and predicated the RNA and proteins binding sites (36,
303 38, 45, 62). To test whether the miRNAs binding ability is required for NS3 inducing miRNA
304 degradation. In the present study, a RPISeq webserver was used to verify those hypotheses
305 (45). The Random Forest (RF) that calculated by RPISeq of indicated miRNAs between each
306 nonstructural protein of JEV was used to evaluate the RPI interaction. The probability
307 threshold of RF used for positive RPIs was higher than 0.5, and only the value of
308 pre-miRNAs or mature miRNAs that interacted with NS3 were all high than 0.5. The mean
309 RF of NS3 with pre-miRNA and mature miRNA was 0.675 and 0.69 respectively, and
310 significantly higher than all the other proteins of JEV (Fig. 6A).

311 To further confirm these *in silico* analysis, an RNA immunoprecipitation (RIP) assay using
312 flag-fused protein to identify the NS3 and miRNA binding affinity, and the fold enrichment of
313 non-structure protein over pcDNA 3.1 was used to determine the RIP value (see material and
314 methods for details). The NS3 has a significantly higher pre-miRNA (i.e., miR-466d-3p,
315 miR-199a-5p, miR-674-5p, miR-574-5p and miR-467a-3p) binding affinity than pcDNA 3.1.
316 In contrast to the NS3, the miRNA binding ability of NS4A was similar to the pcDNA 3.1.
317 However, in contrast to these *in silico* analysis, the RIP assay revealed that the binding ability
318 of pre-miR-466d-3p on NS3 was more than nonuple higher than miR-466d-3p and there is no
319 significant binding affinity of NS3 difference between the pcDNA3.1 and the NS4A (Fig.
320 6B). Furthermore, a CY3 labeled miR-466d-3p mimic revealed that it colocalized with the
321 NS3 expression in the cytoplasm of NA cells (Fig. 6C). However, the miR-466d-3p mimic
322 distributed evenly in the NA cells without the expression of NS3. These results indicate that
323 NS3 specifically binds to pre-miRNA and the binding ability may be essential for miRNA
324 degradation.

325

326 **NS3-mediated the miRNA degradation dependent on arginine rich motifs**

327 Computation methods have been reported to predict the amino acid binding sites of PRIs.
328 However, sequence-based predictors are usually high in sensitivity but low in specificity;
329 conversely structure-based predictors tend to have high specificity, but lower sensitivity (38).
330 In order to combine the advantages of two methods, we used several software to predict RPIs

331 binding sites of NS3. The aaRNA web serve that quantified the contribution of both
332 sequence- and structure-based features, the score of RNA and protein binding specificity was
333 represented as binary propensity (from 0 to 1) (38). The RNA binding sites on NS3 of JEV
334 was analyzed using a PDB format of NS3 (PDB entry 2Z83) with aaRNA, 4 arginine (R202,
335 R226, R388 or R464 respectively) on the NS3 has a higher score (> 0.5) than other amino
336 acid sites (S5 Fig). Furthermore, using two web servers called Pprint and PRIdictor
337 (Protein-RNA Interaction predictor) predicted the amino acid binding sites of PRIs (36, 62),
338 the R202, R226 or R464 that located in the helicase region of NS3 has high miRNA binding
339 ability (S6 Fig and S7 Fig). Similarly, previous studies have reported the arginine as a
340 stronger RNA binding amino acids than others (31). Interestingly, almost all 202, 226, 388 or
341 464 AA sites on the NS3 of flavivirus are arginine but not the hepacivirus or pestivirus. Thus,
342 this sequence alignment confirms the Fig. 3F that only the flavivirus reduce the miRNA
343 expression. When these arginine sites were mutated into other amino acids, the binary
344 propensity or RF value of R202W, R226G and R464Q were all also drop sharply (Fig. 7A
345 and 7B). To further confirm these *in silico* analysis, we constructed 3 substitution mutant
346 vectors of NS3 named as R202W, R226G and R464Q. To investigate the role of miRNA
347 binding affinity in NS3 inducing miRNA turnover, RIP analysis and miRNA expression level
348 were detected in NA cells with these three mutants or parent plasmids of NS3. As shown in
349 Fig. 7C, comparing with the pcDNA 3.1, the mutants of NS3 also specifically bind to
350 pre-miR-466d-3p, and the pre-miR-466d-3p binding ability of R226G and R202W were
351 significantly lower than wild type NS3 (Fig. 7C). Furthermore, the R226G and R202W
352 significantly reduce the degradation of miR-466d-3p, except for R464Q (Fig. 7D). Thus, the
353 202 and 226 arginine of the NS3 contributes to pre-miRNA binding and promotes miRNA
354 degradation.

355

356 Discussion

357 Increasing studies have shown that miRNAs play an important role in the replication and
358 propagation of viruses, including defense of pathogenic viral infections or promotion of viral

359 replication through complex regulatory pathways (26). In the early steps of viral infection,
360 innate viral detected sensors of host cells, such as pathogen recognition receptors (PRRs),
361 recognize a large spectrum of pathogens and initiated a downstream antiviral pathway
362 activates that include miRNAs (65). However, the defense function of host miRNAs can be
363 counteracted by viral suppressors of RNA silencing (VSRs) that inhibit host antiviral
364 responses by interacting with the critical components of cellular RNA silencing machinery or
365 direct participate the host RNA degradation (6, 26). Recently, an increasing number of studies
366 have found that JEV infection were capable of regulating functional miRNAs (3, 14, 53, 60,
367 61, 73). Although plenty of works were examined the host biogenesis regulating by miRNA
368 during the viral infection process, relatively little was known about regulation of miRNA by
369 JEV. Given the importance of miRNA in establishing infection of JEV, our research was aim
370 to explore the interplay between the JEV and host miRNAs.

371 An intriguing aspect of this study is that the JEV globally decrease the host miRNA and
372 independent on Dicer or RISC. We demonstrated that the JEV NS3 could unwind the
373 pre-miR-466d and induce the disfunction of miR-466d-3p. We also observed that the
374 decrement of miR-466d-3p could enhance the JEV replication. Together, these results
375 suggested a role of the miRNA degradation in enhancing JEV replication. Similarly, poly(A)
376 polymerase of vaccina virus (VACV) and 3'UTR of the murine cytomegalovirus degrades the
377 host miRNA via different mechanisms (4, 39). Although this is the first report of a link
378 between the host miRNA degradation and JEV infection, several studies have reported the
379 role of miRNA machineries in the replication of flavivirus, especially dengue virus and
380 Kunjin virus (34, 43). Some analogous systems in degradomes of bacterial and exosomes of
381 eukaryotes are associated with RNA helicases to perform the RNA degradation (22, 49).
382 Hence, the helicase of the JEV seem to be involved in degrading the host miRNA.

383 Why would JEV has evolved a miRNA degradation processing during viral infection in the
384 CNS? There are two possibility why the JEV infection induced global degradation of host
385 miRNA might promote viral infection and replication. First, the host cell encoding miRNA
386 binds to miRNA-binding sites of viral genomes to influence viral function (56, 72).
387 Consistent with this is our observation that restoring miR-466d-3p expression during the JEV
388 infection reduces viral replication. Conversely, the viral induced degradation of host miRNA

389 leads to block the cleavage of viral genome and viral protein. Second, changes in host miRNA
390 expression could also lead to enhance antiviral effector resulting in reducing viral replication
391 (54, 61). However, some viral infections mediate degradation of host miRNAs leading to
392 downstream changes in host transcriptome that can be benefit virus replication and
393 pathogenicity (4). These hypotheses were also confirmed with NS3 of other Flavivirus,
394 including ZIKA could decrease the expression level of miR-466d-3p to enhance the
395 inflammation.

396 Eubacteria, Archaea, and eukaryotes have developed dedicated pathways and complexes to
397 check the accuracy of RNAs and feed undesired RNA into exoribonuclease that degrade
398 RNA. Furthermore, these complexes usually contain adaptor proteins such as RraA (a
399 regulator of RNase E and DEAD-box helicases), RhlB (a DEAD-box RNA helicase) (49),
400 Ski2-like RNA helicases (22, 32) and Suv3 RNA helicase (20). In addition to the RNA
401 degradation, the UPF1 helicase can dissociate miRNAs from their mRNA targets and promote
402 the miRNAs degradation by Tudor-staphylococcal/micrococcal-like nuclease (TSN) (21). The
403 P68 helicase promote unwinding of the human let-7 microRNA precursor duplex, which help
404 let-7 microRNA load into the silencing complex. p72 (DDX17) interact with the
405 Drosha-containing Microprocessor complex and facilitate the processing of a subset of
406 pri-miRNAs in the nucleus and miRNA-guided mRNA cleavage (51). Although host helicase
407 may initially seem to benefit only the cells processing and immune system, multiple studies
408 have shown that viral helicase promote viral replication and disrupt immune system. The NS3
409 of flavivirus are capable of unwinding both the DNA and the RNA that are not of the viral
410 own origin (16, 63). Similar, we observed NS3 of JEV could reduce exogenously constructed
411 ds-miRNA mimic and disable the miRNA mimic.

412 Furthermore, it has been shown that some single-amino-acid mutation in R538, R225 and
413 R268 of DENV, which affect the replication of DENV (17, 58). Another study report that the
414 Asp-285-to-Ala substitution of the JEV NS3 protein abolished the ATPase and RNA helicase
415 activities (63). Furthermore, the helicase domains of NS3 alone is known to induce cell
416 apoptosis in neuron cells (68, 71). These findings suggested that NS3, particularly helicase
417 domain, contributes to the adaptation of flavivirus for efficient replication. Thus, to
418 investigate the details of the NS3 involvement in miRNA degradation, using miRNA deep

419 sequence, we analysis the subtype sequence of mir-466d during the JEV infection and NS3
420 over expression, in which the percentage of incorrect splicing products of mature miRNA was
421 higher than normal. Furthermore, the in vitro unwinding assay demonstrated that the NS3
422 could unwinding the pre-miR-466d and induce the disfunction of miRNA. However, the
423 molecular mechanism of miRNA degradation after unwinding by NS3 need to be clarified in
424 next step experiment.

425 In the present study, we proved that the arginine of NS3 are critical for host pre-miRNA
426 binding and promote the global host miRNA turnover. We constructed 3 arginine variants of
427 NS3 that have single amino acid substitutions on R202W, R226G and R464Q, which were
428 reduced pre-miRNA binding affinity of NS3 and the degradation of miRNA was almost
429 abrogated. Interestingly, these sites are all located in the helicase region of NS3, which from
430 protein sequence of NS3 163 to 619. Similar to our findings, a number of proteins containing
431 arginine-rich motifs (ARMs) are known to bind RNA and are involved in regulating RNA
432 processing in viruses and cells. Such as the ARM of lambdoid bacteriophage N protein or
433 HIV-1 Rev protein bind RNAs and regulate the RNA transport and splicing (23, 57). In
434 addition, four single amino substitution of arginine on HIV-1 Rev protein strongly decrease
435 their RNA binding ability. In brief, these results extend the helicase of Flavivirus function
436 beyond unwinding duplex RNA to the decay of pre-miRNAs, which was provided a new
437 mechanism of helicases in regulating miRNA pathways. Our results suggested that helicase of
438 flavivirus may have the capacity to regulate various cellular miRNAs, which would be a part
439 of a general viral response to overcome host defense mechanisms.

440

441

442

443 **Materials and Methods**

444 **Cell and viruses**

445 The mouse microglia cell line BV-2, the mouse neuroblastoma cell line NA and mouse brain
446 endothelial cell line bEnd.3 were gift from Huazhong Agricultural University which
447 maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo-Fisher, USA)
448 supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) at 37°C in 5%
449 CO₂. The baby hamster Syrian kidney cells line BHK-21 was gift from Huazhong
450 Agricultural University which cultured in Dulbecco's modified Eagle's medium (DMEM,
451 High glucose, Thermo-Fisher, USA) containing 10% FBS. The P3 strain of JEV (Japanese
452 encephalitis virus) was propagated in suckling BALB/c mice (purchased from Vital River
453 Laboratories, China) as previously (37). Briefly, one-day-old suckling mice were inoculated
454 with 10 µl of viral inoculum by the intracerebral (i.c.) route. When moribund, mice were
455 euthanized and brains removed. A 10% (w/v) suspension was prepared by homogenizing the
456 brain in DMEM and centrifuged at 10,000 g for 5 min to remove cellular debris. The brain
457 suspension was filtered through 0.22 µm-pore-size sterile filters (Millipore, USA) and sub
458 packaged at -80°C until further use.

459 **RNA sequencing**

460 Total RNA was extracted from culture NA using the Total RNA Extractor Kit (B511311,
461 Sangon) according to the protocols. A total of 2µg RNA from each sample was used for
462 library preparation according to the manufacturer's instructions of the VAHTSTM
463 mRNA-seq V2 Library Prep Kit for Illumina®. The library fragments were purified with
464 AMPure XP system (Beckman Coulter, Beverly, USA). PCR products were purified
465 (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100
466 system. The libraries were then quantified and pooled. Paired-end sequencing of the library
467 was performed on the HiSeq XTen sequencers (Illumina, San Diego, CA). FastQC (version
468 0.11.2) was used for evaluating the quality of sequenced data. Raw reads were filtered by
469 Trimmomatic (version 0.36) according to protocol. And the remaining clean data was used for
470 further analysis. Gene expression values of the transcripts were computed by StringTie
471 (version 1.3.3b). Principal Component Analysis (PCA) and Principal co-ordinates analysis

472 (PCoA) were performed to reflect the distance and difference between samples. The TPM
473 (Transcripts Per Million), eliminates the influence of gene lengths and sequencing
474 discrepancies to enable direct comparison of gene expression between samples. DESeq2
475 (version 1.12.4) was used to determine differentially expressed genes (DEGs) between two
476 samples.

477 **Plaque assay**

478 JEV was titrated on BHK-21 cells line by viral plaque formation assay as previously (14). A
479 monolayer of BHK cells was co-cultured with JEV (a serial 10-fold dilution prepared in
480 DMEM without FBS) at 37°C. After 2 h incubation, the DMEM containing 3% FBS and 4%
481 sodium carboxymethyl cellulose (CMC) (Sigma) were added to the cells and cultured for 5
482 days. Until the appearance of visible plaques, the cells were fixed with 10% formaldehyde
483 overnight, followed by staining with crystal violet for 2 h. Visible plaques were counted and
484 the viral titers were calculated. All data were expressed as means of triplicate samples.

485 **Transfection of cells with miRNA mimics or inhibitors**

486 Mouse miR-466d-3p mimics, inhibitors, mimics control and negative controls were purchased
487 from GenePharma (China). The sequences of the mimics, inhibitors, mimics control and
488 controls oligo nucleotides were as follows: miR-466d-3p mimics,
489 5'-UAUACAUACACGCACACAUAG-3' (forward) and
490 5'-AUGUGUGCGUGUAUGUAUAAU-3' (reverse); mimic negative controls,
491 5'-UUCUCCGAACGUGUCACGUTT-3' (forward) and
492 5'-ACGUGACACGUUCGGAGAATT-3' (reverse); miR-466d-3p inhibitor,
493 3'-CUAUGUGUGCGUGUAUGUAUA-5'; and inhibitor negative control,
494 3'-CAGUACUUUUGUGUAGUACAA-5'. All of miRNA mimics (1.5 µmol/well), inhibitors
495 (1.5 µmol/well) and control (3 µmol/well) were transfected into BV-2 cells or NA cells (10⁶
496 cells/well) in 12-well plates using Lipofectamine 3000 (Invitrogen) according to the
497 manufacturer's instructions.

498 **Viral infection**

499 BV-2 cells, NA cells or bEnd.3 cells were seeded in 12-well plates (10⁶ cells/well). Until
500 grown to 80% confluence, the cells were incubated with serum-free medium or JEV at a
501 multiplicity of infection (MOI) of 0.1 at 37°C for 2h. After discard of unbound virus and

502 medium, the cells were cultured in 1640 with 10% FBS, 1% Penicillin-Streptomycin Liquid
503 (PS) at 37°C in 5% CO₂ for 48h. Cells and supernatants were collected 48 h post-infection
504 (hpi).

505 **RNA isolation and Reverse transcription real-time PCR.**

506 The total RNA of the cells was isolated by trizol reagent (Thermo Fisher) according to the
507 Manufacturer's recommendations and used for qRT-PCR in an Applied Biosystems® 7500
508 Real-Time PCR Systems (Thermo fisher) as described previously. For the evaluation of
509 mRNA, to obtain cDNA, the total RNA (1 µg) was reverse transcribed by PrimeScript RT
510 reagent Kit with gDNA Eraser (Takara, China). The IL-1β and components of RISC mRNA
511 expression levels of the NA cells were quantified with the SYBR Green qPCR kit (Takara) by
512 following the manufacturer's instruction using gene-specific primers (S2 Table).
513 Amplification was performed at 95°C for 30 s ,95°C for 5 s, 60°C for 34 s, followed by 40
514 cycles of 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 15 s. The expression of
515 IL-1β was normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
516 expression using the 2-ΔΔCT method as previously (69). For the expression of miRNA, the
517 reverse transcription primers of all miRNA were added with specific stem-loop structure at
518 the end of 5' (S3 Table). The pri-, pre- and mature-miRNA levels of cells were quantified
519 with the SYBR Green qPCR kit using miRNA sequence-specific primers (S4 Table and S5
520 Table). Amplification was performed at 95°C for 30 s ,95°C for 5 s, 60°C for 34 s, followed
521 by 40 cycles of 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 15 s. The relative
522 levels of microRNAs were normalizing to U6 determined by the 2-ΔΔCT method as
523 previously (73).

524 **ELISA**

525 The culture supernatants of the experimental group and control group cells or brain tissue
526 lysates were collected and stored at -80°C. The protein levels of IL-1β in cell cultures or
527 mouse brain tissue lysates were determined by enzyme-linked immunosorbent assay (ELISA)
528 kits (eBioscience) according to the manufacturer's instructions.

529 **Immunoprecipitations**

530 Immunoprecipitation with FLAG fused protein was performed as described previously (70).
531 Briefly, BSR cells was transfected with the FLAG-tagged plasmid. At 48 hpi, the cells

532 expressing FLAG fusion protein were harvested and lysed with CelLytic™ M lysis buffer
533 (Sigma) containing protease inhibitor cocktail (Sigma) in 4°C for 30 minutes on a shaker at
534 10 rpm. Each cell lysate was incubated with ANTI-FLAG M2 affinity gel (Sigma) at 4°C
535 overnight on a shaker at 10 rpm. Then, the agarose gel was centrifuged for 30 seconds at 8200
536 g to remove the supernatants. After three times washed with 0.5 ml of TBS, the bound
537 proteins were eluted by boiling with SDS-PAGE loading buffer for 5 min and determined
538 with Western blotting.

539 **Western blotting**

540 The cells were lysed with RIPA Lysis and Extraction Buffer (Thermo Scientific), and the
541 protein level was measured with the Enhanced BCA Protein Assay kit (Sigma-Aldrich). The
542 extracts which contained 25 µg of total proteins were subjected to 10% SDS polyacrylamide
543 gel, and protein blots were transferred to Nitrocellulose membrane (NC) after electrophoresis.
544 The membrane was then washed with TBST and blocked in 5% skimmed milk at 4°C
545 overnight. All primary antibodies were prepared at a dilution of 1:1,000 in 1% bovine serum
546 albumin (BSA) in 1×PBST, followed by horseradish peroxidase-conjugated secondary
547 antibodies (Sigma) for 1h at room temperature. Blots were detected by enhanced
548 chemiluminescence reagent (Thermo Scientific) and developed by exposure in Tanon 5200
549 (Tanon) using Tanon MP software. In addition, α -tubulin played the role of an internal
550 control.

551 **RNA immunoprecipitation (RIP)**

552 RIP was performed as previously (48), with slight modification. Briefly, NA cells was
553 transfected with the FLAG-tagged NS3, FLAG-tagged NS2B or FLAG-tagged pcDNA 3.1
554 pcDNA3.1 plasmid for 48h. 10^7 cells expressing FLAG fusion protein were harvested with
555 2.5% trypsin and resuspended in 5ml PBS. 143µl of 37% formaldehyde was added to the
556 resuspension to cross-link for 10 minutes on a shaker, and 685µl of 2M the glycine was used
557 to block the formaldehyde. The cells were washed twice by 5ml ice-cold PBS and centrifuged
558 for 2 minutes at 400 g to collect the cells pellet. 1ml CelLytic™ M lysis buffer (Sigma)
559 containing 20µl 0.1M Phenylmethylsulfonyl fluoride, 20µl protease inhibitor cocktail (Sigma)
560 and 5µl 40U/µl RNase inhibitor (Invitrogen) was added to cells, and the cells were kept on ice
561 and sonicated for 2 mins (10 sec on, 10 sec off, Amplitude 15 µm) until the lysate is clear.

562 Followed by centrifuging the lysate for 3 minutes at 14000g to collect supernatant. Each 1ml
563 cell lysate was added to the 40µl washed with ANTI-FLAG M2 affinity gel (Sigma) at 4°C
564 for 4h on a shaker at 10 rpm. The next step was to centrifuge the resin for 30 seconds at 8200
565 g and remove the supernatants. After the resin was washed three times with 0.5 ml 500µl of
566 TBS, the total RNA was extracted with Trizol reagent and analyzed by RT-qPCR.
567 The fold change of each RIP reaction from RT-qPCR data was calculated via $2^{-\Delta\Delta CT}$
568 method as previously with minor modification (42) and the formulas see below. All the Ct
569 value of each specimen (FLAG-tagged NS3, FLAG-tagged NS2B or FLAG non-fused blank
570 pcDNA3.1) was normalized with the Input RNA to eliminate the possible differences in RNA
571 samples preparation (ΔCt). To obtain the $\Delta\Delta Ct$, the normalized experimental RIP fraction
572 value (ΔCt) was normalized to unspecific background as an internal control (ΔCt normalized
573 of pcDNA3.1 sample). Finally, the $\Delta\Delta Ct$ [Experimental/pcDNA3.1] was performed with
574 linear conversion to calculate the Fold enrichment. The formula of Fold enrichment is shown
575 below.

$$576 \quad \Delta Ct = Ct[RIP] - (Ct[Input] - \log_2(\text{Input/RIP dilution factor}))$$

$$577 \quad \Delta\Delta Ct[\text{Experimental/pcDNA3.1}] = \Delta Ct[\text{Experimental}] - \Delta Ct[\text{pcDNA3.1}]$$

$$578 \quad \text{Fold enrichment} = 2^{(-\Delta\Delta Ct[\text{Experimental/pcDNA3.1}])}$$

579

580 **MicroRNA target prediction**

581 The sequences for the miRNA whose expression changed during JEV Infection have been
582 submitted to the public miRNA database miRBase (www.mirbase.org). The miR-466d-3p
583 target binding sites in the 3'UTRs of mouse gene transcripts were identified with Target Scan
584 software (<http://www.targetscan.org/>).

585 **Bioinformatics Analysis of the changed miRNA**

586 The total RNA was isolated from mouse brains with Trizol reagent (Invitrogen) for miRNA
587 and mRNA Microarray. miRNA and mRNA hybridization were performed by shanghaiBio
588 Corporation (shanghai, China) with the use of 8×15 K Agilent Mouse microRNA
589 Microarray and 4×44 K Agilent Whole Mouse Genome Oligo Microarray. For each sample

590 pair, the experiments were done with two independent hybridizations for miRNA (Agilent's
591 miRNA Complete Labeling and Hyb Kit) or for mRNA (Cy3 and Cy5 interchanging
592 labeling). Hybridized arrays were scanned at 5 μ m resolution on a Microarray Scanner
593 (Agilent p/n G2565BA). Data extraction from images was done by using Agilent Feature
594 Extraction (FE) software version 9.5.3. The mRNAs which were caused significant changes
595 (change fold \geq 2.0, p value $<$ 0.05) by JEV infection in mouse brain were clustered using GO
596 and KEGG enrichment tools (ShanghaiBio Analysis System). And the interaction of the most
597 significantly differential expression proteins was retrieved by STRING (59).

598 **Construction of mutant NS3 plasmids**

599 The plasmid encoding FLAG-tag E, C, PrM, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and
600 NS5 cDNA clone in pcDNA3.1 (+) flanked by Kpn I ribozyme and Xba I ribozyme
601 sequences as described previously (70). The AA residues at R202W, R226G R464Q of NS3
602 in the SA strain were swapped individually or both by overlap extension PCR as described
603 previously. Briefly, in a PCR reaction 1 μ l of full-length NS3 gene cDNA was mutated and
604 amplified with 20 μ M each of 202, 226 or 464 site mutation forward and reverse primers and
605 20 μ M each of JEV-NS3 forward and reverse primers using PrimeSTAR GXL DNA
606 Polymerase (TAKARA) according to the manufacturer's instructions. The size of PCR
607 products was 1.86 kb and the products were purified by gel purification kit. The PCR mixture
608 was heated at 94 $^{\circ}$ C for 2 min, followed by 35 cycles of amplification at 98 $^{\circ}$ C for 10 s, 55 $^{\circ}$ C
609 for 30s and 68 $^{\circ}$ C for 1min45s, and a final extension at 68 $^{\circ}$ C for 10 min. All NS3 fragments
610 with FLAG-tag that included AA mutations and NS3 cDNA vector were digested with
611 enzyme sets KpnI and XbaI (Thermo Scientific). Following the digestions, the NS3 fragments
612 with FLAG-tag and NS3 cDNA vector were ligated together at an approximate molar ratio of
613 1:3 using TaKaRa DNA Ligation Kit LONG (TAKARA) according to the manufacturer's
614 instructions.

615 **The integrative analysis of miRNAs and mRNAs**

616 Usually, there were more than hundreds target genes among each miRNA used 3 miRNA
617 target predication databases TargetScan (1), miRDB (67) and microRNA (5). The integrative
618 analysis of miRNAs and mRNAs allowed us to predict the major target of the decreasing
619 miRNAs. To accurately elucidate the correlation between the mRNA expression pattern and

620 miRNA targeted regulation, mRNA expression files were measured with mRNA microarray
621 to predict the major decreasing miRNAs in mice brain. Firstly, those abnormal miRNAs
622 target mRNAs were used to find out the common mRNA (with a threshold of fold change \geq
623 2.0, p value < 0.01) from the mRNA expression profile and miRNA expression profile. Using
624 those common genes as seed, a protein-protein interaction network was constructed to
625 discover the core gene of miRNA target. A total of 42 interacting proteins with 177
626 interactions were retrieved from the STRING database (protein-protein interaction enrichment
627 p value $< 1.0e-15$). The k-Means clustering algorithm was applied to segregate the network of
628 those interacting proteins into different subgroup.

629 **LC-MS analysis**

630 The FLAG fused NS3 was purified by immunoprecipitation and washed with pure
631 water twice in 0.5 ml Eppendorf tube. The following procedure to digest was
632 performed as previously described (33). To dehydrate, the specimen was treated with
633 50% acetonitrile for 30 min send following with 100% acetonitrile for another 30 mi.
634 After dehydration, the gel was dried in SpeedVac concentrator (Thermo Savant,
635 Holbrook, NY, USA) for 30 min then restored with reduction buffer (25mM
636 NH_4HCO_3 in 10mM DTT) at 57°C for 1 hafted removing reduction buffer, the gel
637 was dehydrated again with 50% and 100% acetonitrile respectively for 30 min. To
638 rehydrate, the gel was removed acetonitrile and incubated with 10 μL 0.02 $\mu\text{g}/\mu\text{L}$
639 trypsin in 25mM NH_4HCO_3 for 30 min at RT. For tryptic digestion, 20 μL cover
640 solution (25mM NH_4HCO_3 in 10% acetonitrile) was added for digested 16 hours at
641 37°C, and the supernatants were transferred into another tube. For peptide extraction,
642 the gel was extracted with 50 μL extraction buffer (5% TFA in 67% acetonitrile) at
643 37°C for 30min. Finally, the peptide extracts and the supernatant of the gel were
644 combined and then completely dried in Speed Vac concentrator. The specimen was
645 analyzed by the direct nanoflow liquid chromatography tandem mass spectrometry
646 (LC-MS/MS) system and the ion spectra data were identified in the protein database.

647 **Ethics Statement**

648 The experimental infectious studies were performed in strict accordance with the Guide for

649 the Care and Use of Laboratory Animals Monitoring Committee of Hubei Province, China,
650 and the protocol was approved by the Scientific Ethics Committee of Huazhong Agricultural
651 University (protocol No. Hzaumo-2015-018). All efforts were made to minimize the suffering
652 of the animals.

653 **Statistical analysis**

654 All experiments were performed at least three times with similar results. The data generated
655 were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). For all tests,
656 the P value of <0.05 was considered significant.

657

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- 889
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- 891

892 Figure Captions

893 **FIG 1. JEV infection downregulates global miRNA expression in mice nervous system.**

894 (A) Changes in miRNA expression upon JEV infection of mice brain. The 4-week-old
895 BALB/c mice was infected with 10^3 PFU of P3 or medium control. After 9 days infection, the
896 miRNA expression level of JEV-infected mice was compared with those from the
897 non-infected control. The color scale is based on \log_2 changes in expression. (B) Changes in
898 miRNA expression upon JEV infection of NA cells. The NA cells was infected with 0.1 MOI
899 and 0.01 MOI of P3 or medium control. After 48 hours infection, the miRNA expression level
900 of JEV-infected cells was compared with those for the non-infected control. The color scale is
901 based on \log_2 changes in expression. (C) Western blot of NA cells infected with 0.1 MOI and
902 0.01 MOI of P3 or medium control after 48 hours. (D) Virus titer of NA cells infected with
903 0.1 MOI and 0.01 MOI of P3 after 48 hours. (E) Quantitative RT-PCR (qRT-PCR) analyses
904 of the mature miRNA levels in the BV2, NA and bEnd.3 cells after 48 hours JEV infection (1
905 MOI). For all graphs, results are shown as mean \pm SD. Significance was assessed using a
906 Student's t test, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

907

908 **FIG 2. JEV infection induce incorrect processing of host miRNAs.**

909 pre-miR-466d-3p processing and types of mature miRNAs (miR-466d-3p and miR-466h-3p).
910 The blue arrow represented the miR-466h-3p located in the pre-miR-466d and the red arrow
911 represented the miR-466d-3p located in the pre-miR-466d. (B) Analysis of expression level of
912 mature miR-466d-3p, miR-466h-3p, pre-miR-466d-3p or pri-miR-466d using the quantitative
913 RT-PCR (qRT-PCR) in in NA or BV2 cells at indicated MOI of P3 after 48 hours infection.
914 (C) Quantification of exogenous miR-466d mimic degradation in NA cells by qRT-PCR. The
915 NA cell was infected with P3 at MOI of 0.1 and transfected with miR-466d-3p at 36 hpi.
916 After 48 hours infection, the total RNA from the NA cells was used to quantitative analysis.
917 For all graphs, results are shown as mean \pm SD. Significance was assessed using a Student's t
918 test, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

919

920 **FIG 3. NS3 of JEV-mediated degradation of mir-466d in neuron cells.**

(A) Transcriptional

921 and translation inhibition assay. The NA cells was infected with P3 at MOI of 0.1 and the
922 T-705, α -Amanitin or CHX were treated with NA cells at 12 hpi or 42 hpi respectively. After
923 48 hours infection, the total RNA from the NA cells was used to quantify the expression level
924 of miR-466d-3p. (B) Relative analyses of miR-466d-3p expression level in NA cells that
925 transfected with indicated expression plasmid with/without JEV infection. After 48 hours
926 transfection/infection, the total RNA from NA cells was used to quantify the relative
927 expression level of miR-466d-3p (VS pcDNA 3.1 control) by qRT-PCR. (C) Changes in
928 miRNA expression upon transfection of NS3 in the NA cells. The NA cells was transfected
929 with NS3 or pcDNA 3.1 control. After 48 hours transfection, the miRNA expression level of
930 NS3-transfected cells was measured by RNA deep sequencing and compared with those for
931 the pcDNA 3.1-transfected control. The color scale is based on \log_2 changes in expression.
932 (D) qRT-PCR analyses of the mature miRNA levels in NA cells after 48 hours transfection of
933 NS3. (E) The qRT-PCR analyses of the human miRNA in NA cells after 48 hours
934 transfection with NS3 or infection with P3 at 0.1 MOI. (F) The qRT-PCR analyses of the
935 miR-466d-3p in NA cells after 48 hours transfection with NS3 of ZIKA virus, WNV,
936 DENV1, DENV2, CSFV, BVDV and HCV. (G) Quantification of exogenous miR-466d
937 mimic degradation in NA cells by qRT-PCR. The NA cell was transfected with NS3, and
938 after 48 hours the NA cells was transfected with miR-466d-3p mimic. After 54 hours
939 transfection of NS3, the total RNA from the NA cells was used to quantitative analysis. For
940 all graphs, results are shown as mean \pm SD. Significance was assessed using a Student's t test,
941 * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

942

943 **FIG 4. Block miR-466d enhance IL-1 β secretion and promote JEV replication.** (A) The
944 homogenization of mice brain was collected at 9 dpi and the expression level of IL-1 β in mice
945 brain was determined by ELISA. (B) The cell supernatant from NA cells was collected at
946 indicated infection time point and infection dose the expression level of IL-1 β was determined
947 by ELISA. (C) Introduction of miR-466d-3p binding sites in IL-1 β , NS3, NS5 and E. The
948 miR-466d-3p complementary sequences to the coding sequence of IL-1 β , NS3, NS5 and E are
949 indicated as bold and italic. (D-G) The synthetic mimic of miR-466d-3p decreased the
950 expression of IL-1 β and the block the replication of JEV, and the inhibitor of miR-466d-3p

951 has the opposite effect. The NA or BV2 cells was infected/treated with JEV at MOI of 0.01,
952 LPS 100ng/ml or medium control, respectively. After 48 hours infection/treatment, the cells
953 was transfected with mimic of miR-466d-3p or inhibitor of miR-466d-3p. (D) After 6 hours
954 transfection, the total RNA from NA cells was used to quantify the relative expression level
955 of miR-466d-3p (VS negative control) by qRT-PCR. (E) After 6 hours transfection, the total
956 RNA from NA cells was used to quantify the relative expression level of IL-1 β (VS negative
957 control) by qRT-PCR. (F) After 6 hours transfection, the supernatant of cells was collected to
958 quantify the expression level of IL-1 β (VS negative control) by ELISA. (G) After 24 hours
959 transfection, the supernatant of cells was collected to determine the titer of JEV. (H) The
960 miR-466d target sites-fused GFP was co-transfected with NS3 or/and miR-466d-3p mimic,
961 after 48 hours transfection the cells were stained with DAPI. The fluorescence was observed
962 under the fluorescence microscope. For all graphs, results are shown as mean \pm SD.
963 Significance was assessed using a Student's t test, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

964

965 **FIG 5. Unwinding of host miRNA by NS3 block the silencing function of miRNA.** (A)
966 Schematic of the synthetic double strand pre-miR-466d-3p mimic and double strand
967 miR-466d-3p mimic. The sequence of miR-466d-3p in pre-miR-466d-3p was highlighted in
968 bold. (B-C) The synthetic double strand pre-miR-466d-3 and double strand mimic of
969 miR-466d-3p were unwound by purified NS3 generated from NA cells. The flag-tagged NS3
970 was expressed in the NA cells and purified by the affinity gel. The indicated concentrations of
971 NS3 (0, 1, 3 and 5 $\mu\text{g/ml}$) was incubated with 20 pmol of miR-466d-3p mimic (B) or
972 pre-miR-466d-3 mimic (C). After 2 hours incubation at 37 $^{\circ}\text{C}$, the miR-466d-3p mimic or
973 pre-miR-466d-3 mimic was determined by 10% native polyacrylamide gel electrophoresis.
974 (D) The miR-466d-3 mimic and pre-miR-466d-3 mimic were degraded after the unwinding
975 with NS3. After 24 hours transfection, the total RNA of NA cells was used to quantify the
976 relative expression level of miR-466d-3p (VS negative control) by qRT-PCR. (E-F) The
977 unwinding miR-466d-3p mimic by NS3 could not decrease the expression of IL-1 β . The total
978 RNA of NA cells was used to quantify the relative expression level of IL-1 β (VS negative
979 control) by qRT-PCR (E) and the supernatant of NA cells was used to determine the
980 expression level of IL-1 β by ELISA (F). For all graphs, results are shown as mean \pm SD.

981 Significance was assessed using a Student's t test, *p≤0.05, **p ≤0.01 and ***p ≤0.001.

982

983 **FIG 6. NS3 has specific binding affinity of pre-miRNA.** (A) The pre-miRNA and mature

984 miRNA binding affinity of indicated non-structure protein of JEV was predicated with a

985 webserver named RPISeq (<http://pridb.gdcb.iastate.edu/RPISeq/>) and the Random Forest (RF)

986 that calculated by RPISeq was used to evaluated the RPI. The probability threshold of RF

987 used for positive RPIs was higher than 0.5. (B) The pri-, pre- and mature miRNA level of

988 miR-466D-3p, miR-199a-5p, miR-674-5p, miR-574-5p and miR-467a-3p were selected to

989 determine binding affinity between the NS3 and miRNA by RIP. The NA cells was

990 transfected with plasmid of FLAG-tagged NS3, NS4A or pcDNA 3.1, and these

991 FLAG-tagged proteins was purified by affinity gel after 48 hours transfection. After

992 purification, the total RNA on the FLAG-tagged protein was used to quantify the relative

993 binding affinity of indicated pri-, pre- and mature miRNA (VS pcDNA 3.1 control) by

994 qRT-PCR. The fold change of each RIP reaction from qRT-PCR data was calculated as Fold

995 enrichment= $2^{-(\Delta \Delta Ct[\text{Experimental} / \text{pcDNA } 3.1])}$, $\Delta \Delta Ct[\text{Experimental}/\text{pcDNA } 3.1]=$

996 $\Delta Ct[\text{Experimental}]-\Delta Ct[\text{pcDNA } 3.1]$, $\Delta Ct=Ct[\text{RIP}]-(\text{Ct}[\text{Input}]-\log_2(\text{Input}/\text{RIP}$

997 $\text{factor}))$. (C) The pre-miR-466d-3p mimic and NS3 were colocalized in the NA cells. After 48

998 hours transfection, the CY3 labeled miR-466d-3p mimic and NS3 stained with FITC in NA

999 cells were detected by fluorescence microscope. Images are representative of three

1000 independent experiments. For all graphs, results are shown as mean ± SD. Significance was

1001 assessed using a Student's t test, *p≤0.05, **p ≤0.01 and ***p ≤0.001.

1002

1003 **FIG 7. NS3-mediated the miRNA degradation dependent on arginine rich motifs.** (A-B)

1004 The RNA binding sites on the NS3 were predicted with 3 webserver (supplement) and 3 of

1005 arginine has higher RNA binding affinity than others. The sequence of these predicted RNA

1006 binding sites was changed to other amino acid as indicated and evaluated the RNA binding

1007 affinity by binary propensity (A). After changed all three AA sites (R202W, R226G and

1008 R464Q), the probability threshold of RF was evaluated. The probability threshold of RF used

1009 for positive RPIs was higher than 0.5 (B). (C) The pri-, pre- and mature miR-466-3p binding

1010 affinity of the substitution mutant of NS3 was determined by the RIP assay. Three
1011 substitution mutant vectors of NS3 named as R202W, R226G and R464Q was constructed by
1012 overlap extension PCR. After 48 hours transfection, the FLAG-tagged NS3 was purified with
1013 affinity gel and the total RNA on the NS3 was used to quantify the relative binding affinity of
1014 pre-miR-466-3p (VS pcDNA 3.1 control) by qRT-PCR. The fold change of each RIP reaction
1015 from qRT-PCR data was calculated as $\text{Fold enrichment} = 2^{-(\Delta \Delta C_t[\text{Experimental} / \text{pcDNA 3.1}])}$. (D)
1016 Relative analyses of miR-466d-3p expression level in NA cells that transfected with indicated
1017 expression plasmid. After 48 hours transfection, the total RNA from NA cells was used to
1018 quantify the relative expression level of miR-466d-3p (VS pcDNA 3.1 control) by qRT-PCR.
1019 For all graphs, results are shown as mean \pm SD. Significance was assessed using a Student's t
1020 test, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

1021

1022 **Supporting Information Legends**

1023 **S1 Fig. The protein interaction network was constructed by STRING that based on the**
1024 **candidate genes (42 genes) of common mRNA from the miRNAs and mRNA expression**
1025 **profile.** Light blue lines, indicated interactions from curated databases. Purple lines,
1026 experimentally validated interactions. Green lines, predicted interactions from gene
1027 neighborhoods. Red lines, predicted interactions from gene fusions. Dark blue lines, predicted
1028 interactions from gene co-occurrence. Yellow lines, represented interactions from textmining.
1029 Light purple lines, interactions from protein homology. Blank lines, interactions from
1030 co-expression. And the dotted lines indicated interactions of bridge different subnetworks.
1031 The colored nodes in the network showed the query proteins and first shell of interactors. The
1032 network is divided into many groups according to biological Process (GO). The red nodes in
1033 the network represented the genes which played a role in the immune and inflammation
1034 process. Average number of connections per node is 2.81 (protein-protein interaction
1035 enrichment p value $< 1.0e-15$).

1036

1037 **S2 Fig. The mature miRNA sequence-specific reads were determined by deep**
1038 **sequencing of the 18–24 nt fraction of JEV-infected or NS3-expressed cells.** The mature

1039 miR-466d-3p, miR-381-3p, miR-466a-3p, miR-467a-3p, miR-199a-5p and miR-466h-3p
1040 sequence-specific reads were determined by deep sequencing of the 18-24 nt fraction of
1041 P3-infected cells, or NS3 transfected cells. The bold type sequence represents the correct
1042 sequence of the miRNA. The percentage of indicated sequence reads was analyzed by the
1043 miRDeep2.

1044

1045 **S3 Fig. Phylogenetic tree and alignment based on the full-length NS3 sequences of 37**
1046 **Flaviviride virus strains.** Phylogenetic tree deduced from a full-length alignment of NS3
1047 from indicated viruses using the neighbor-Joining method as implemented in MEGA7 (Left).
1048 The numbers below the branches are bootstrap values for 1000 replicates. Phylogenetic
1049 analysis of 37 NS3 gene nucleotide sequence from Flaviviride included JEV vaccine strain
1050 SA-14-14-2 (GenBank No. M55506.1), lineage II WNV strain (GenBank No. M12294.2),
1051 Dengue virus 1 isolate TM100 (GenBank No. KU666942.1), Dengue virus 2 isolate TM26
1052 (GenBank No. KU666944.1), Zika virus strain MR 766 (GenBank No. AY632535.2),
1053 Classical swine fever virus C-strain (GenBank No. Z46258.1), Chikungunya virus isolate
1054 MY/08/4567 (GenBank No. FR687343.1) Hepatitis C virus QC69 subtype 7a (GenBank No.
1055 EF108306.2) and Bovine viral diarrhea virus 1 isolate MA_101_05 (GenBank No.
1056 LT968777.1). (Right) Alignment of amino acid sequences of NS3 of 37 Flaviviride virus. The
1057 four conserved amino acids are highlighted in yellow and the arginine is highlighted with blue
1058 spot.

1059

1060 **S4 Fig. qRT-PCR analysis of Dicer1, AGO1, AGO2, Tmr1, Tsn and Gemin4.** Analysis of
1061 major components gene expression of Dicer1, AGO1, AGO2, Tmr1, Tsn and Gemin4 using
1062 qRT-PCR in NAs infected with the indicated P3 after 48 hours.

1063

1064 **S5 Fig. Predication of RNA-binding sites in NS3 amino acid sequence using aaRNA.** The
1065 RNA binding sites on NS3 of JEV was analyzed using a PDB format of NS3 (PDB entry
1066 2Z83) with aaRNA (<https://sysimm.ifrec.osaka-u.ac.jp/aarna/>), 4 arginine (R202, R226, R388
1067 or R464 respectively) on the NS3 has a higher score (> 0.5) than other amino acid sites.

1068

1069 **S6 Fig. Predication of RNA-binding sites in NS3 amino acid sequence using Pprint.** The
1070 R202 and R464 that located in the helicase region of NS3 has high RNA interacting ability,
1071 which was analyzed by aaRNA (<http://www.imtech.res.in/raghava/pprint/>).

1072

1073 **S7 Fig. Predication of RNA-binding sites in NS3 amino acid sequence and miRNA**
1074 **nucleotide sequences using PRIdictor.** The R202 and R226 that located in the helicase
1075 region of NS3 has high RNA binding ability, which was analyzed by PRIdictor
1076 (<http://bclab.inha.ac.kr/pridictor/>).

1077

1078 **S1 Table. The proteins of NA cells identified by LC-MS from the**
1079 **co-immunoprecipitation with NS3.**

1080

1081 **S2 Table. Primers used for quantification of IL-1 β and components of RISC mRNA.**

1082

1083 **S3 Table. The reverse transcription primers used for reverse transcribed all mature**
1084 **miRNA that were added with specific stem-loop structure at the end of 5'.**

1085

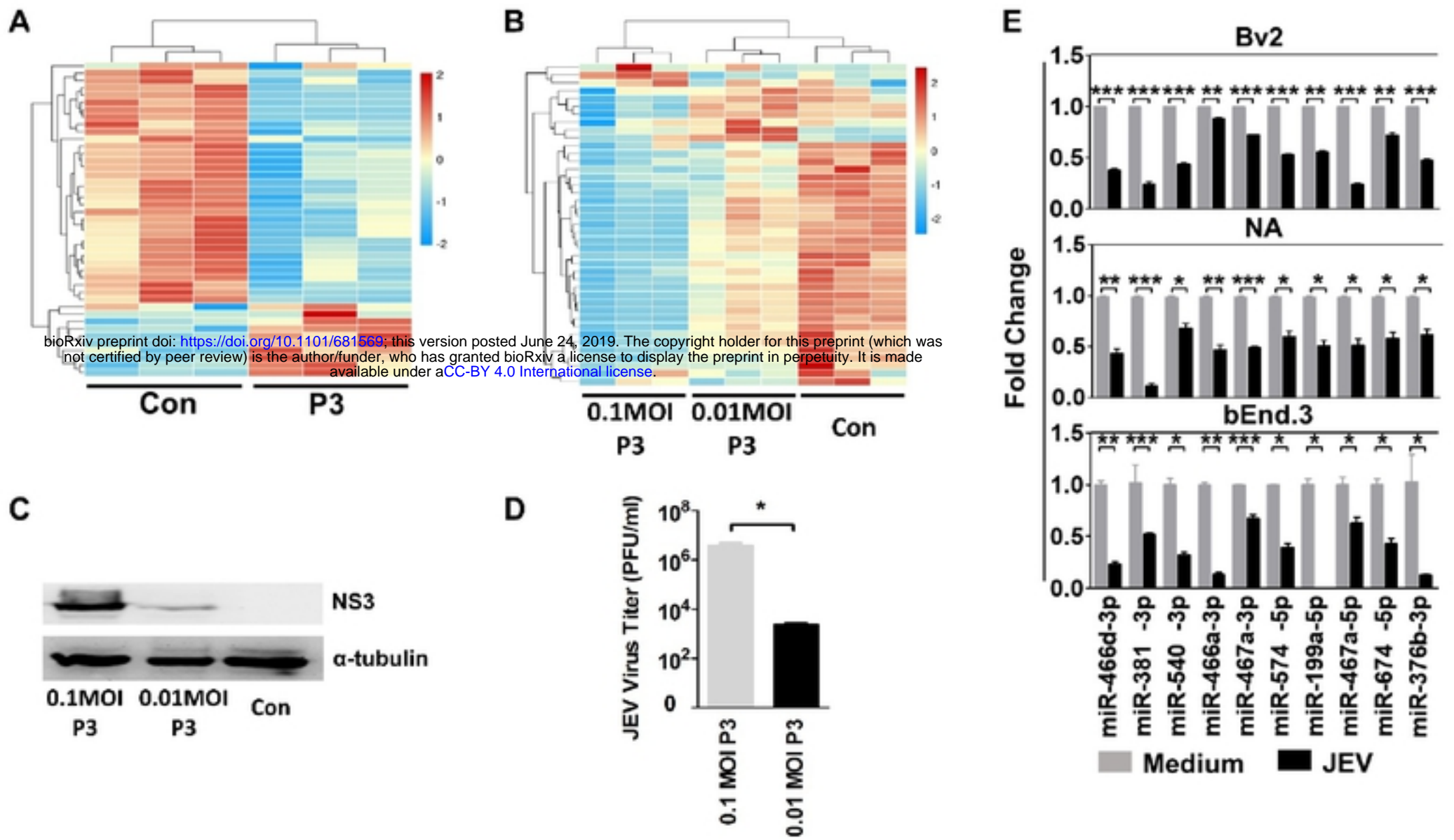
1086 **S4 Table. Primers used for quantification of pri-miRNA and pre-miRNA.**

1087

1088 **S5 Table. Primers used for quantification of mature miRNA and U6.**

1089

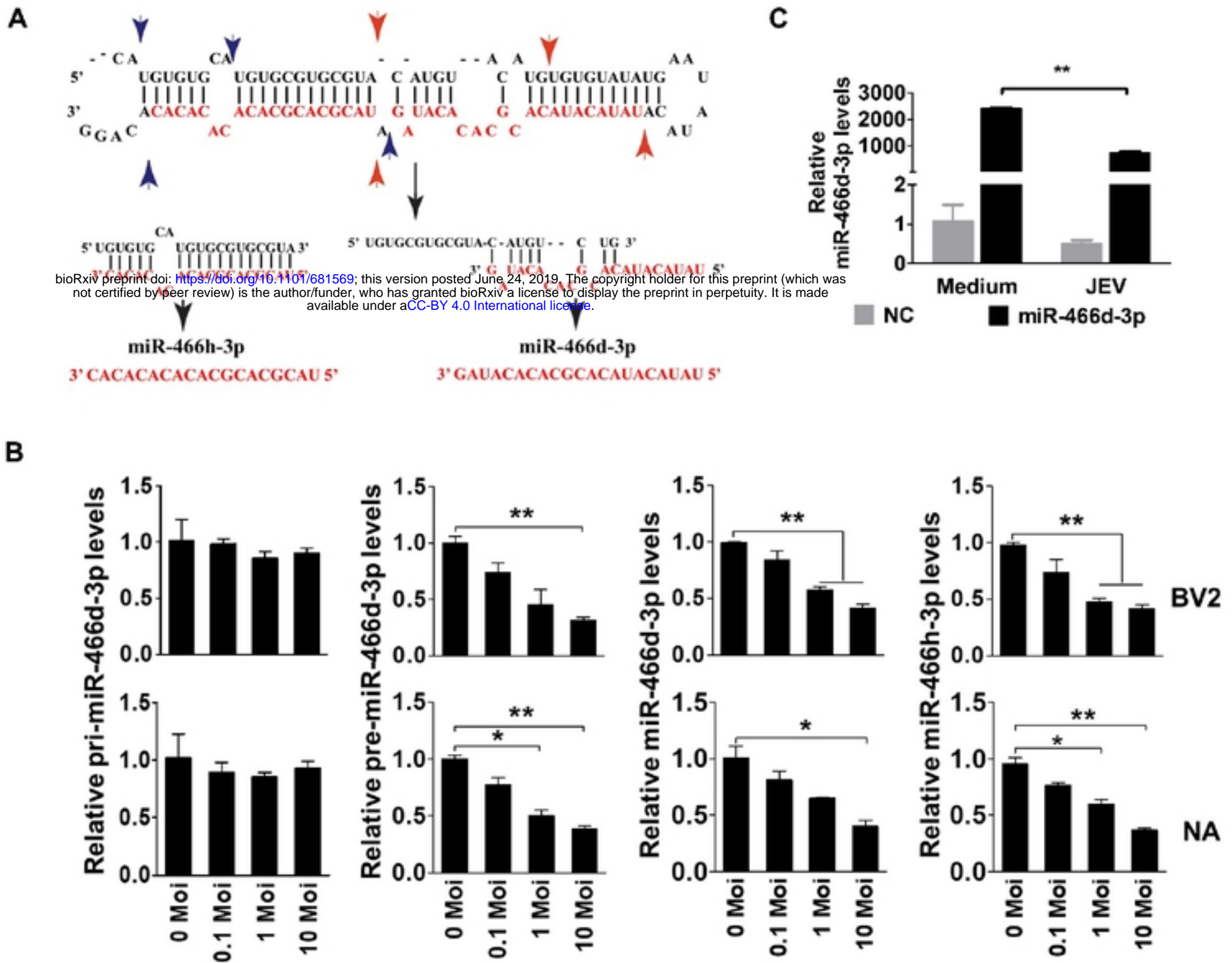
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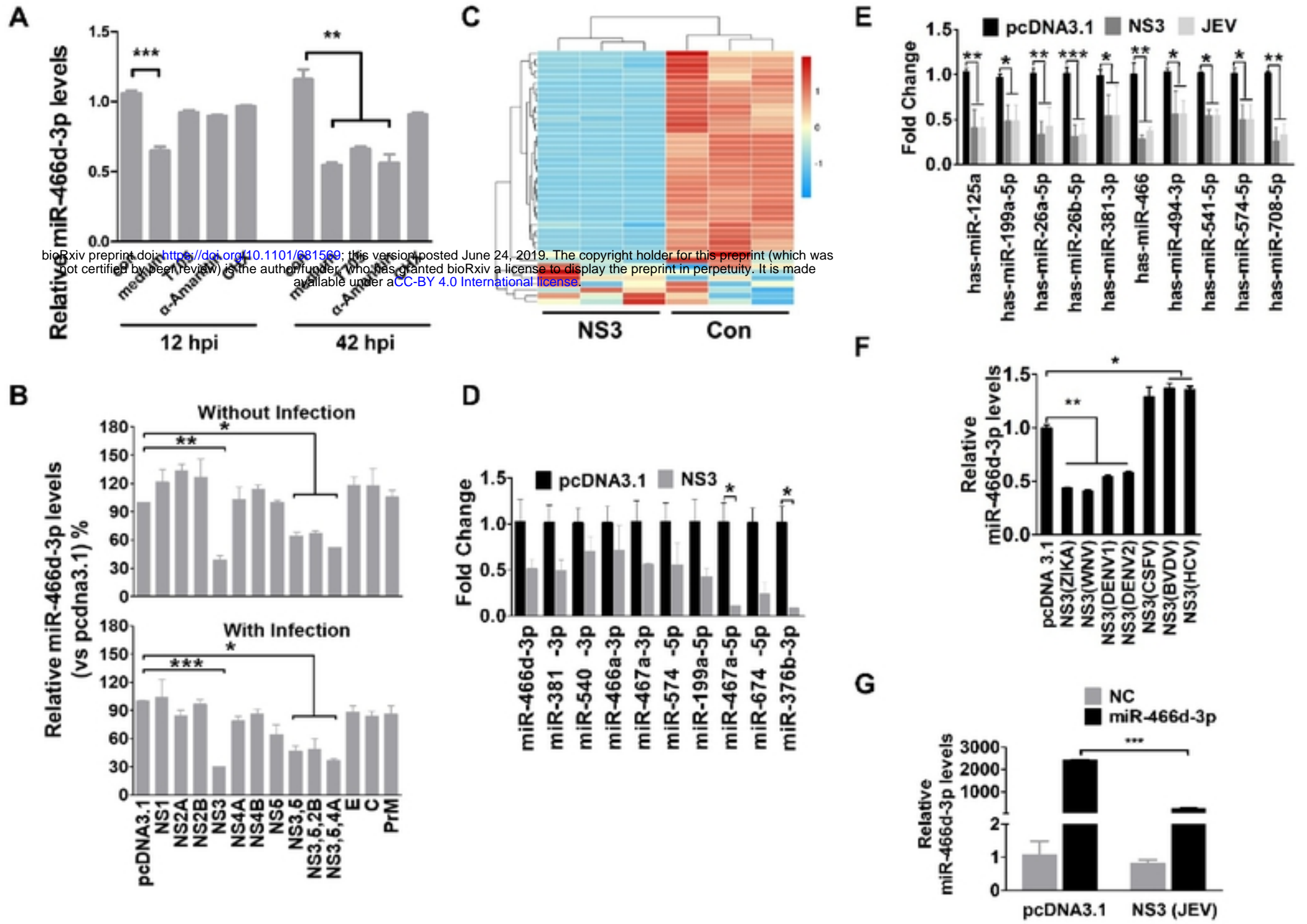
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3

4 Fig 2.

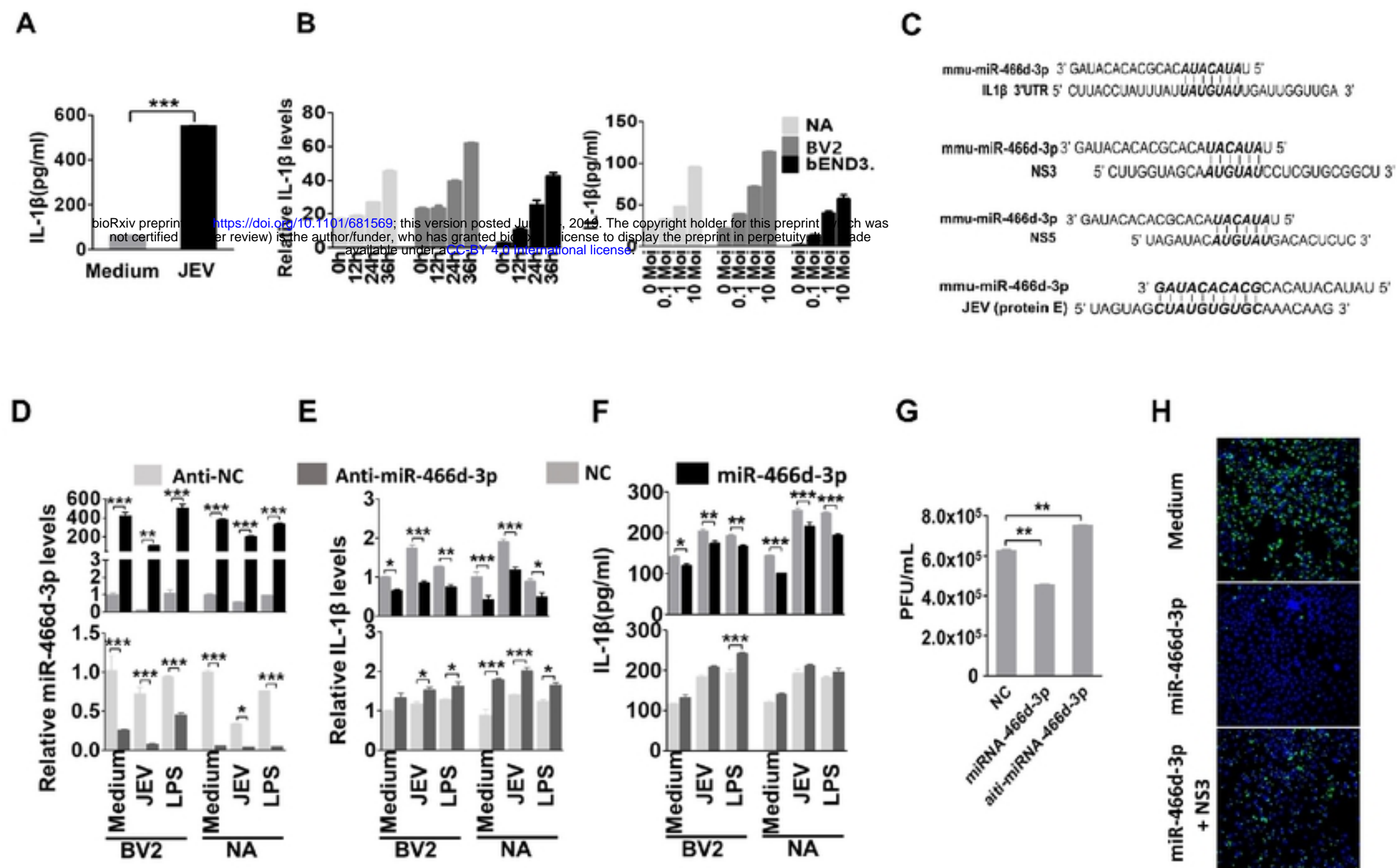


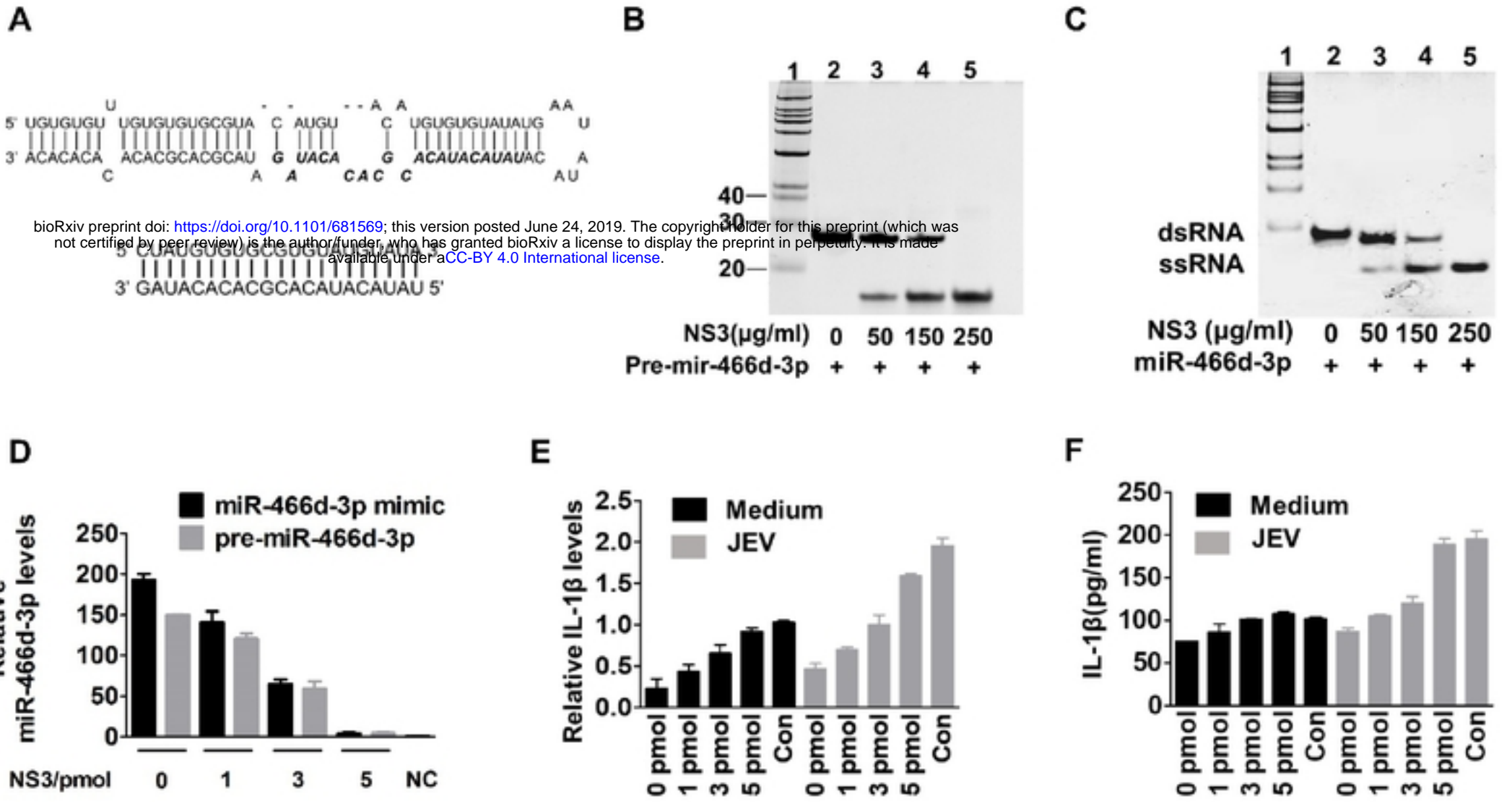
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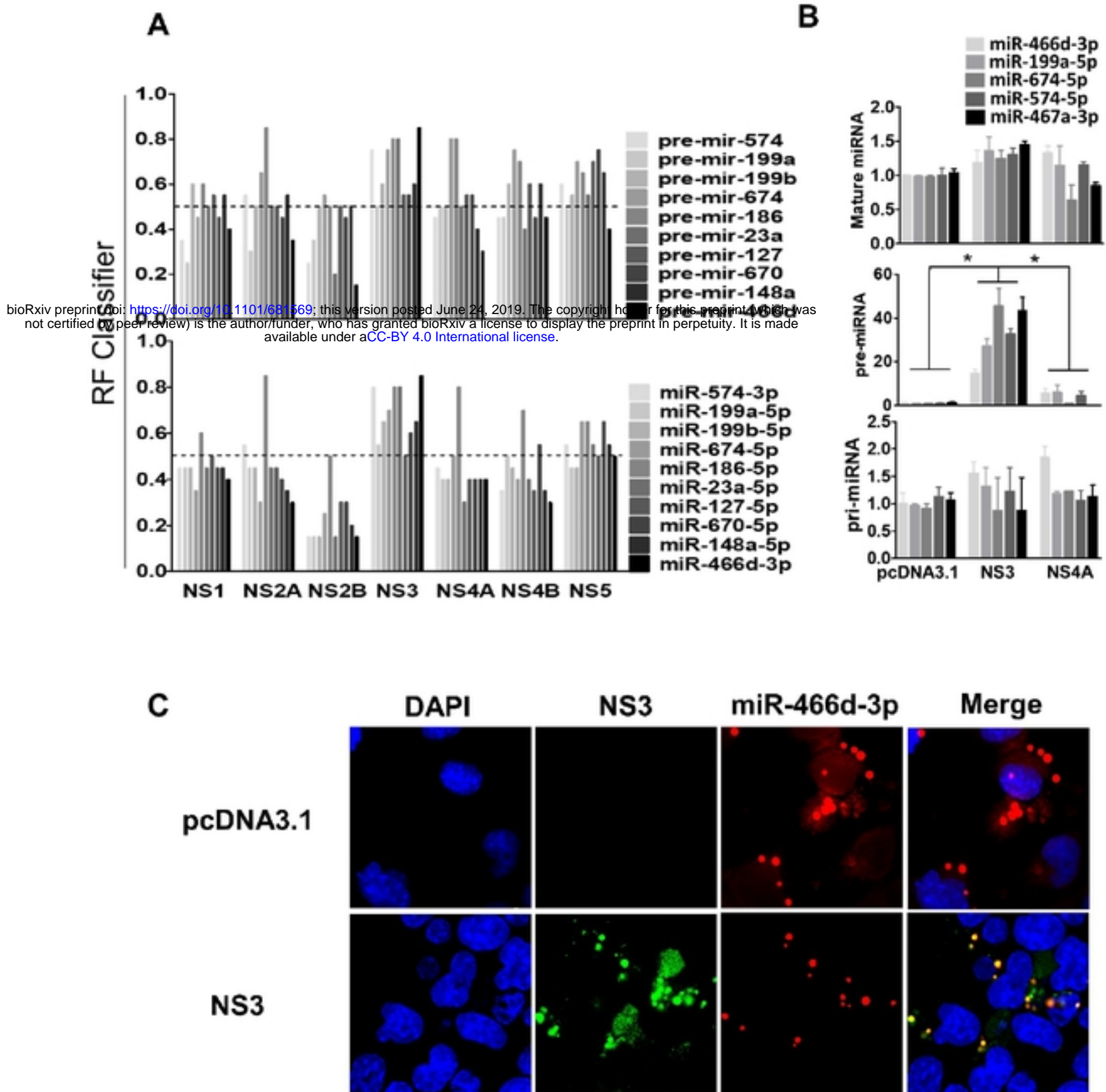


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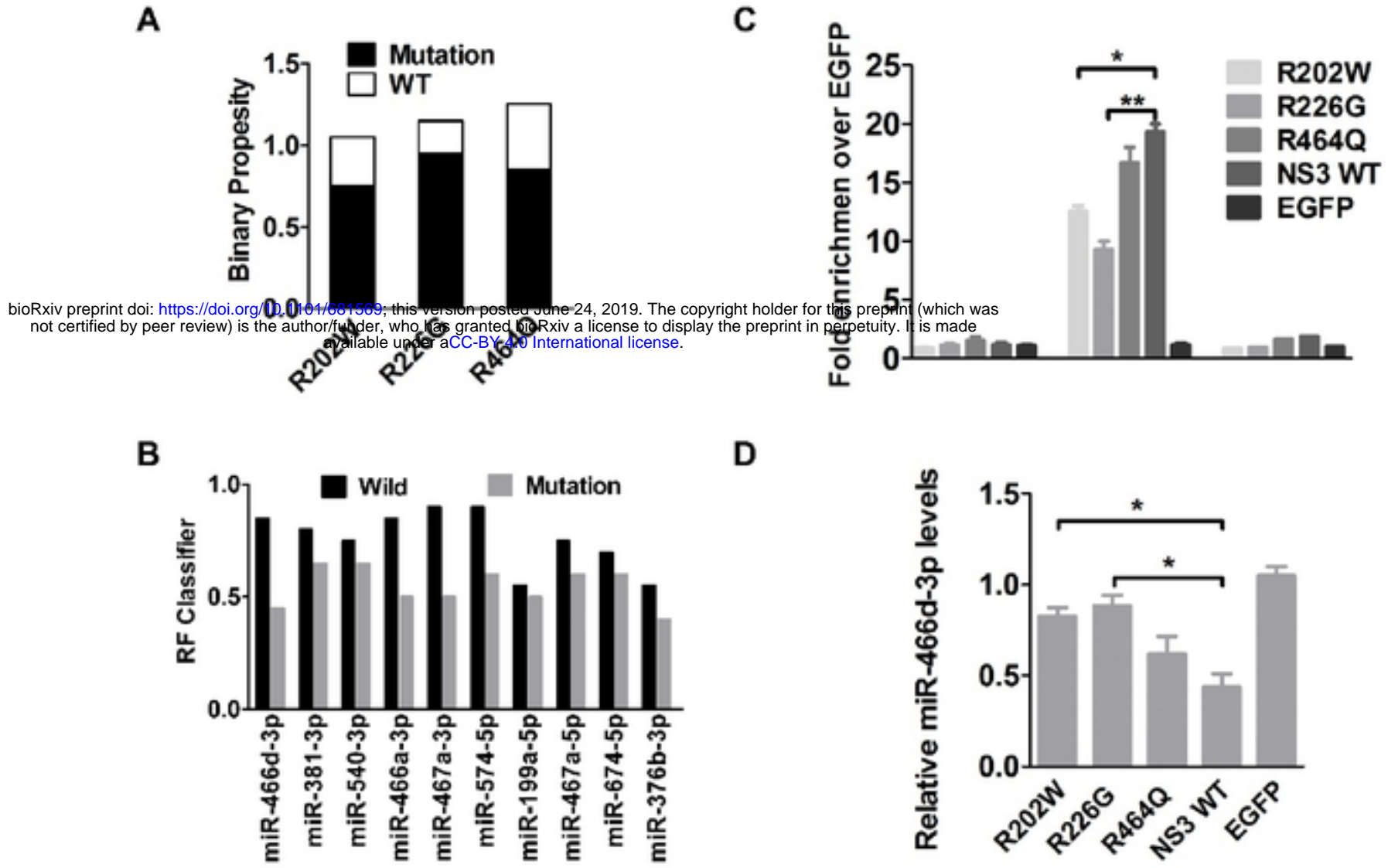
8 Fig 4.







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