

1 Cooperativity between the 3' untranslated region microRNA binding sites is critical for  
2 the virulence of eastern equine encephalitis virus

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27 Short Title: miRNA binding sites in EEEV 3' UTR

28 **Abstract**

29 Eastern equine encephalitis virus (EEEV), a mosquito-borne RNA virus, is one of the  
30 most acutely virulent viruses endemic to the Americas, causing between 30% and 70%  
31 mortality in symptomatic human cases. A major factor in the virulence of EEEV is the  
32 presence of four binding sites for the hematopoietic cell-specific microRNA, miR-142-  
33 3p, in the 3' untranslated region (3' UTR) of the virus. Three of the sites are “canonical”  
34 with all 8 seed sequence residues complimentary to miR-142-3p while one is “non-  
35 canonical” and has a seed sequence mismatch. Interaction of the EEEV genome with  
36 miR-142-3p limits virus replication in myeloid cells and suppresses the systemic innate  
37 immune response, greatly exacerbating EEEV neurovirulence. The presence of the  
38 miRNA binding sequences is also required for efficient EEEV replication in mosquitoes  
39 and, therefore, essential for transmission of the virus. In the current studies, we have  
40 examined the role of each binding site by point mutagenesis of seed sequences in all  
41 combinations of sites followed by infection of mammalian myeloid cells, mosquito cells  
42 and mice. The resulting data indicate that both canonical and non-canonical sites  
43 contribute to cell infection and animal virulence, however, surprisingly, all sites are  
44 rapidly deleted from EEEV genomes shortly after infection of myeloid cells or mice.  
45 Finally, we show that the virulence of a related encephalitis virus, western equine  
46 encephalitis virus, is also dependent upon miR-142-3p binding sites.

47

48 **Author Summary**

49 Eastern equine encephalitis virus (EEEV) is one of the most acutely virulent mosquito-  
50 borne viruses in the Americas. A major determinant of EEEV virulence is a mammalian  
51 microRNA (miRNA) that is primarily expressed in myeloid cells, miR-142-3p. Like  
52 miRNA suppression of host mRNA, miR-142-3p binds to the 3' untranslated region  
53 (UTR) of the EEEV genome only in myeloid cells suppressing virus replication and the  
54 induction of the innate immune response. In this study, we used point mutations in all  
55 four miR-142-3p binding sites in the EEEV 3' UTR to understand the mechanism behind  
56 this miRNA suppression. We observed that decreasing the number of miR-142-3p  
57 binding sites leads to virus escape and ultimately attenuation *in vivo*. Furthermore,  
58 another virus, western equine encephalitis virus, also encodes miR-142-3p binding sites  
59 that contribute to virulence *in vivo*. These results provide insight into the mechanism of  
60 how cell-specific miRNAs can mediate suppression of virus replication.

61

62 **Introduction**

63 Eastern equine encephalitis virus (EEEV) is a mosquito-borne alphavirus that  
64 causes severe manifestations of encephalitis in humans resulting in high mortality rates  
65 and long-term neurological sequelae in symptomatic cases [1] and is one of, if not the  
66 most acutely virulent virus circulating in North America. Even though both EEEV and  
67 the closely related Venezuelan equine encephalitis virus (VEEV) cause encephalitic  
68 disease, they exhibit drastic differences in their pathogenesis, in large part due to  
69 differential infectivity of the viruses for myeloid cells. VEEV is highly myeloid cell tropic  
70 and induces a robust innate immune response while EEEV is largely replication  
71 defective in these cells, which limits the production of systemic IFN- $\alpha/\beta$  and other innate  
72 cytokines *in vivo* [2]. Critical to EEEV virulence, a host microRNA (miRNA), miR-142-3p,  
73 primarily expressed in hematopoietic cells [3], prevents translation of the EEEV genome  
74 and virus replication in myeloid cells by interaction with binding sites in the EEEV 3'  
75 untranslated region (UTR) [4]. Remarkably, although mosquitoes do not express miR-  
76 142-3p, presence of the miR-142-3p binding site sequences is required for efficient  
77 mosquito infection and, presumably, transmission of EEEV in nature [4].

78 miRNAs are short ~22 nucleotide long non-coding RNAs that can be transcribed  
79 from both coding and non-coding chromosomal regions. miRNAs bind to complimentary  
80 sequences in host mRNA as part of the RNA-induced silencing complex (RISC) through  
81 the seed sequence, nucleotides 2-8 at the 5' end of the miRNA [5]. Perfect  
82 complementarity throughout the entire miRNA and target mRNA can lead to  
83 degradation of the mRNA, but is rarely seen in mammalian interactions [6,7]. More  
84 commonplace in mammalian cells is a perfect match (canonical sequence) or a single

85 mis-match (non-canonical sequence) in the seed sequence and the target mRNA [8,9]  
86 leading to translational repression, downregulation of protein expression, and RNA  
87 destabilization [10,11]. The miR-142-3p blockade virtually eliminates EEEV translation  
88 and replication in myeloid cells [4].

89 miRNAs can interact with the 5' UTR (e.g. hepatitis C virus (HCV) [12]), 3' UTR  
90 (e.g. EEEV [4], and bovine viral diarrhea virus (BVDV) [13]) or coding regions (e.g.  
91 influenza [14-16], and enterovirus-71 [17,18]) (reviewed in [19]). This interaction leads  
92 to either inhibition of virus translation and replication (e.g. EEEV [4]) or stabilization of  
93 the virus RNA and increased replication (e.g. HCV [20] and BVDV [13]). There are  
94 three canonical and one non-canonical miR-142-3p sites in a 260 nt stretch of the EEEV  
95 3' UTR [4]; however, the contribution of each site to replication inhibition is not known,  
96 and the contribution of individual or combinations of miRNA binding sites to virulence in  
97 animals is not known for any virus.

98 In the current studies, we have leveraged the highly restrictive effect of miR-142-  
99 3p on EEEV replication *in vitro* and *in vivo* to examine the impact of each of the four  
100 sites present in the 3' UTR on virus replication and disease. We have used point  
101 mutations to alter each of the miRNA binding sites to a non-functional sequence, either  
102 individually or in combination. We then examined infection efficiency for myeloid cells or  
103 mouse lymphoid tissue, replication, innate immune response induction, disease severity  
104 after mouse infection and competence for mosquito cell replication. We found that both  
105 canonical and non-canonical sites contributed to the virulence phenotype of wild type  
106 (WT) EEEV with a single site exerting the most potent effect upon virulence. The  
107 contribution of all sites to restriction of myeloid cell replication was reinforced by the

108 observation that deletion mutations in the 3' UTR arose rapidly during WT EEEV  
109 replication *in vitro* and in mice removing all of the miR-142-3p sites and conferring  
110 myeloid cell tropism to EEEV. Finally, we provide evidence that another encephalitic  
111 alphavirus, western equine encephalitis virus (WEEV) also possesses functional miR-  
112 142-3p binding sites in its 3' UTR.

113 **RESULTS**

114 *miR-142-3p binds to the EEEV 3' UTR within the RISC complex in RAW cells*

115        The RNA induced silencing complex (RISC) facilitates repression of host mRNA  
116    and viral RNAs through interactions of Argonaute protein (Ago) with host miRNAs  
117    [20,21]. In an effort to determine if miR-142-3p bound to viral RNA and targeted it to the  
118    RISC complex, we utilized a translation reporter that encodes the 5' UTR and the WT  
119    EEEV 3' UTR encoding the four miR-142-3p binding sites (Figure 1A) or the 11337  
120    deletion mutant 3' UTR that lacks all of the miR-142-3p binding sites [4] and two  
121    different experimental methodologies: 1) immunoprecipitation of the Ago protein to  
122    isolate RNAs interacting with the complex and 2) precipitation of biotin-labeled miR-142-  
123    3p to ascertain whether miR-142-3p interacted directly with the EEEV 3' UTR in myeloid  
124    cells. Virus-specific RT-PCR of precipitated nucleic acids was then used to quantify the  
125    level of reporter RNA.

126        After electroporation of the EEEV translation reporters into RAW 264.7  
127    monocyte/macrophage cell line, which constitutively expresses miR-142-3p[4], WT  
128    EEEV RNA was ~15 fold higher in the Ago-immunoprecipitated fraction compared to  
129    11337 reporter RNA when normalized to mock electroporated cells and total input RNA  
130    (Figure 1B) demonstrating that WT EEEV interacted with the Ago complex. Next, to  
131    determine specifically if miR-142-3p bound to the EEEV 3' UTR, we electroporated a  
132    biotin-labeled miR-142-3p or biotin-labeled scrambled control miRNA into RAW cells  
133    along with the EEEV reporters and immunoprecipitated RNA complexes with  
134    streptavidin beads. We detected ~18 fold higher levels of WT EEEV RNA in lysates that  
135    were co-transfected with a biotin-labeled miR-142-3p mimic compared to

136 electroporation with a scrambled miRNA mimic (Figure 1C). Furthermore, we detected  
137 only very low levels of 11337 reporter RNA in lysates after co-transfection with both the  
138 biotin-miR-142-3p mimic or scrambled mimic demonstrating the specificity of miR-142-  
139 3p interaction with the WT EEEV 3' UTR. PCR analysis indicated that the initial levels of  
140 WT and 11337 reporter RNA in electroporated RAW cells were comparable prior to  
141 immunoprecipitation. Thus, the EEEV 3' UTR interacts with miR-142-3p inside RISC  
142 complex through the Ago proteins presumably leading to translational inhibition of the  
143 EEEV genome.

144

145 *Combinatorial mutation of the EEEV miR-142-3p binding sites increases virus*  
146 *replication in myeloid cells*

147 A higher number of miRNA binding sites within a virus 3' UTR may lead to  
148 increased suppression of tissue-specific virus replication and virus attenuation *in vivo*  
149 [22,23]. To determine the contribution of each miR14-2-4p binding site to restriction of  
150 EEEV replication, we created point mutant EEEV viruses in every combination of one,  
151 two, three or all four miR-142-3p binding sites disrupted in the 3' UTR (Figure 1D) in the  
152 background of a nLuc expressing virus [24]. We incorporated mutations at 3 nucleotides  
153 at positions 2 (C2G), 4 (T4C), and 6 (A6T) in each of the canonical miR-142-3p binding  
154 sites that is complimentary to the miR-142-3p seed sequence (Figure 1D, sites 1, 3 and  
155 4). We also made similar mutations in the non-canonical miR-142-3p binding site (site  
156 2), except at position 2 where an A-G mutation was inserted making all four binding  
157 sites have the same 3 nucleotide mutations. This yielded fifteen mutant viruses with the

158 combined four-site mutant containing a total of 12 point mutations over a span of ~250  
159 nucleotides.

160 In BHK-21 fibroblast cells, which do not express miR-142-3p [4], point mutation  
161 of the miR-142-3p binding sites did not significantly change virus replication compared  
162 to WT EEEV or the 11337 deletion mutant at 12 hpi (Figure 2A) or subsequent time  
163 points (data not shown). In RAW cells, a monocyte/macrophage cell line which express  
164 an intermediate level of miR-142-3p, and C57BL/6 bone marrow-derived dendritic cells  
165 (BMDCs), which express a high level of miR-142-3p [4], mutation of each miR-142-3p  
166 binding site individually, leaving three intact miR-142-3p binding sites, did not  
167 significantly increase virus replication compared to WT EEEV (Figure 2B-C). Mutation of  
168 any two of the miR-142-3p binding sites resulted in a reproducible but small increase in  
169 virus replication in both RAW and BMDCs that did not attain statistical significance  
170 *versus* WT EEEV. However, mutation of 3 miR-142-3p binding sites, leaving only a  
171 single binding site intact, led to significantly higher levels of virus replication at 12 hpi in  
172 both RAW and BMDC cells (Figure 2B-C), with the exception of the virus with mutations  
173 in sites 1, 2, and 3 leaving site 4 intact in RAW cells.

174 Both the 11337 deletion mutant and the 1234 point mutant are impaired in their  
175 ability to replicate in *in vitro* in C6/36 mosquito cells and in mosquito vectors [4],  
176 suggesting a requirement for these 3'UTR sequences in the EEEV transmission cycle.  
177 Therefore, we used the point mutant viruses to determine whether or not the individual  
178 miR-142-3p binding sites contribute to EEEV replication in mosquito cells. At 8 hpi,  
179 replication of both the quadruple mutant 1234 and 11337 mutant viruses were  
180 significantly reduced in C6/36 cells compared to EEEV WT (Figure 2D). Of the three

181 mutation viruses, only mutants 134 and 234 were significantly reduced compared to  
182 EEEV WT, while there was no difference in replication of the other EEEV mutants. By  
183 12hpi, only mutants 1234 and 11337 were significantly reduced compared to WT  
184 (Figure 2E). Combined with the fact that the 11337 mutant was significantly more  
185 inhibited than the four site point mutant at 12 hpi ( $p<0.0001$ , unpaired t test), this result  
186 suggests that the individual miR-142-3p binding site sequences may not be the primary  
187 elements in the 3' UTR that promote mosquito replication, but potentially, a sequence-  
188 dependent RNA secondary structure or other specific sequence is critical.

189 Together, these data demonstrate that while mutations in the miR-142-3p binding  
190 sites do not alter virus replication in non-miR-142-3p expressing cells, mutations in 3 of  
191 the miR-142-3p binding sites results in EEEV replicative escape from miR-142-3p  
192 suppression in myeloid cells. Therefore, 2 or more miR-142-3p binding sites are  
193 required for significant suppression of EEEV myeloid cell replication *in vitro*  
194 demonstrating that cooperativity between the miR-142-3p binding sites enhances EEEV  
195 suppression.

196

197 *Combinatorial mutation of the EEEV miR-142-3p binding sites increases virus*  
198 *attenuation in mice.*

199 We have previously demonstrated that deletion of all of the miR-142-3p binding  
200 sites (virus 11337) resulted in virus attenuation *in vivo* due to increased myeloid cell  
201 replication and systemic IFN- $\alpha/\beta$  production [4]. Since decreasing the number of  
202 functional miR-142-3p binding sites resulted in increased myeloid cell replication *in vitro*,  
203 we sought to determine whether a similar phenomenon could be observed *in vivo*. In

204 CD-1 mice infected with viruses lacking a single miR-142-3p binding site (single  
205 mutants), only mutation of site 4 lead to a significant increase in survival compared to  
206 WT EEEV (Figure 3A); there were no significant survival differences with mutation of  
207 sites 1, 2, or 3 alone. Mutation of two miR-142-3p binding sites (double mutants)  
208 resulted in greater attenuation and higher percent survival compared to the single  
209 mutants; however, virus attenuation was dependent on the combination of sites that  
210 were mutated. The virus with sites 3 and 4 mutated (34) had the highest survival rate of  
211 the double mutants and was significantly attenuated with 50% survival compared to WT  
212 EEEV (Figure 3A). Percent survival was also significantly higher in mice infected with  
213 the 13, 14, and 23 viruses compared to WT EEEV but lower than the 34 mutant. There  
214 was no significant survival difference in mice infected with the 12 and 24 mutant viruses  
215 compared to WT EEEV.

216 Infection of mice with viruses encoding three mutant miR-142-3p binding sites  
217 and only one functional miR-142-3p binding site (triple mutants) resulted in significant  
218 survival differences for all of the mutants compared to WT EEEV (Figure 3A).  
219 Additionally, percent survival for the mutant viruses 134 and 234 was higher than that of  
220 the double mutants, but not to the level of 11337 or the quadruple mutant 1234 virus.  
221 Finally, mice infected with the mutant 1234 virus was not significantly different than  
222 11337. We also observed similar survival percentages of the miR-142-3p binding site  
223 mutants in inbred C57BL/6 mice compared to CD-1 mice (S1 Figure).

224 Together these results demonstrate that, similar to our data on virus replication in  
225 myeloid cells, reduction in the number of miR-142-3p binding sites leads to increased  
226 survival and higher virus attenuation *in vivo*. Importantly, none of the 1, 2, or 3 site point

227 mutants were similarly attenuated to the four-site mutant 1234 or 11337, suggesting  
228 each of the four miR-142-3p binding sites, including the non-canonical site (site 2)  
229 contributes to the fully virulent phenotype of the WT virus. There was little significant  
230 difference in attenuation between viruses with similar numbers of mutations. Mutant  
231 virus 4 was significantly attenuated compared to mutant 1 ( $P<0.05$ ) and mutant 34 virus  
232 was significantly attenuated compared to 12 ( $P<0.01$ ), which may suggest a higher  
233 impact of site 4 than other sites on virulence *in vivo*. Yet overall, the data indicate that  
234 each miR142-3p binding site contributes to EEEV virulence.

235

236 *Early virus replication in popliteal lymph nodes and inflammatory response is dependent*  
237 *on the number of miR-142-3p binding sites*

238 We also previously demonstrated that the miR-142-3p binding site mutant,  
239 11337, rescued virus replication in the popliteal lymph nodes (PLN) *in vivo* early after  
240 infection compared to WT EEEV [4]. Therefore, we next determined whether or not  
241 combinatorial mutation of the miR-142-3p sites led to differences in virus replication in  
242 the PLNs early after infection. CD-1 mice were infected with the nLuc-expressing EEEV  
243 mutant viruses and the PLNs were harvested 12 hpi, and virus replication was  
244 quantified by nLuc analysis. In the PLNs, the deletion mutant 11337 virus had the  
245 highest level of virus replication at 12 hpi (Figure 3B), which was significantly higher  
246 than WT. Replication of the four site mutation virus, 1234, and the triple mutant viruses  
247 were higher than either the single or double mutant viruses, but lower than the 11337  
248 mutant virus. All of the triple mutant viruses except mutant 123 had significantly higher  
249 levels of virus replication in the PLN compared to WT although that mutant exhibited a

250 trend toward higher replication. Of the double mutants, only mutants 34 ( $P<0.05$ ) had  
251 significantly higher replication than WT. Replication of the single site mutation viruses  
252 was not significantly different from the WT, although the site 4 mutant exhibited a trend  
253 towards higher replication evidenced in some animals. These results are similar to the  
254 *in vitro* results in myeloid cells in that the mutant viruses with 2 or 3 functional miR-142-  
255 3p binding sites showed restricted viral replication. In summary, elimination of 3 miR-  
256 142-3p binding sites conferred myeloid cell replication leading to higher virus levels of  
257 nLuc in the PLN. Virus replication *in vivo* was more variable after infection than in  
258 cultured myeloid cells suggesting that other factors potentially including variability in the  
259 expression of miR-142-3p in PLN cells or numbers of myeloid cells could be influencing  
260 PLN replication.

261 Myeloid cell replication by the mutant 11337 virus leads to IFN- $\alpha/\beta$  production *in*  
262 *vivo* by 12 hpi [4]. We hypothesized that decreasing the number of miR-142-3p binding  
263 sites would lead to higher levels of systemic IFN- $\alpha/\beta$  contributing to the virus attenuation  
264 that is seen *in vivo*. At 12 hpi, serum IFN- $\alpha/\beta$ , measured by bioassay, was undetectable  
265 in WT and single mutation viruses with the exception of two mice infected with the site 4  
266 mutant (Figure 3C). This is consistent with the trend for site 4 mutant viruses towards  
267 higher PLN replication and significant attenuation *in vivo* demonstrating that activity of 3  
268 miR-142-3p binding sites largely suppresses IFN- $\alpha/\beta$  production except when the miR-  
269 142-3p binding site (site 4) is closest to the poly (A) tail. Serum IFN- $\alpha/\beta$  levels in mice  
270 infected with the double mutant viruses was dependent on the particular combination of  
271 mutations. Mutant viruses 12 and 14 only had a single mouse with detectable levels of  
272 IFN- $\alpha/\beta$ . Viruses with mutations in sites 23, and 24 led to a higher number of mice with

273 serum IFN- $\alpha/\beta$ , but the average for the groups was not significantly different from WT.  
274 Mutant 13 and 34 were the only double mutant viruses to have significantly higher levels  
275 of serum IFN- $\alpha/\beta$  compared to WT ( $P<0.01$ ). For the triple mutants, mutant 123 and 234  
276 didn't not induce significantly higher levels of IFN- $\alpha/\beta$  compared to WT.

277 By 24 hpi (Figure 3D), we detected serum IFN- $\alpha/\beta$  in some mice infected with the  
278 WT, and more mice infected with the single mutants and the double mutants than at  
279 12hpi. These data suggest there is a delayed induction of IFN- $\alpha/\beta$  with these viruses  
280 compared to 11337 and the other mutants. It is possible that the increased IFN- $\alpha/\beta$   
281 production by WT may be due to changes in miR-142-3p levels within the PLN as a  
282 response to infection, leading to virus replication or, possibly, virus escape of miR-142-  
283 3p suppression in myeloid cells due to the presence of higher levels of WT virus in the  
284 PLN at 24 hpi compared to 12 hpi [25].

285 Early production of cytokines and chemokines produced by myeloid cells can  
286 influence trafficking of immune cells and the induction of the adaptive immune response  
287 [26,27]. A single PLN was harvested at 12 hpi from each of three CD-1 mice infected  
288 with either WT, 11337 or mock-infected and RNA was isolated for qRT-PCR to quantify  
289 cytokine and chemokine mRNA levels using a panel of cytokine and chemokines that  
290 are involved in trafficking and induction of the adaptive immune response, specifically  
291 trafficking of immune cells to peripheral tissues during infection [26]. Significantly higher  
292 mRNA levels of the inflammatory cytokines *Ifnb*, *Ifng*, *Il6*, and *Il1b* and chemokines  
293 *Cxcl10*, *Ccl3*, and *Ccl2* were detected in 11337-infected PLN compared to WT-infected  
294 PLN. (S2A Figure). For both *Ccl4* and *Cxcl1*, statistical significance was influenced by

295 several WT-infected mice that had high mRNA levels in the PLN, but overall WT-  
296 infected mice had lower levels compared to 11337-infected mice.

297 To further examine the relationship of myeloid cell replication and the number of  
298 miR-142-3p binding sites to induction of chemokine mRNAs within the PLN, we  
299 quantified the level of *Cxcl10* mRNA within the PLN after infection with each of EEEV  
300 mutants at 12 hpi (S2B Figure). Similar to IFN- $\alpha/\beta$  levels in the serum, *Cxcl10* mRNA  
301 levels in the PLN depended on the number of miR-142-3p binding sites. Mice infected  
302 with WT and the single mutants had similar levels of *Cxcl10* mRNA within their PLN.  
303 Mice infected with the double mutant virus 34 had significantly ( $P<0.001$ ) higher levels  
304 of *Cxcl10* mRNA compared to WT. Mutant 123 of the triple mutant viruses didn't not  
305 significantly increase *Cxcl10* mRNA within the PLN, but all of the other triple mutant  
306 viruses, the quadruple mutant 1234, and 11337 had significantly higher levels of *Cxcl10*  
307 mRNA. Together, our results demonstrate the increasing myeloid cell replication leads  
308 to not only higher levels of serum IFN- $\alpha/\beta$ , but also higher mRNAs levels of  
309 inflammatory cytokines and chemokines in the PLN. Overall, these results demonstrate  
310 that, similar to myeloid cells *in vitro*, the four miR-142-3p binding sites in the EEEV 3'  
311 UTR have a cumulative effect upon PLN replication and response, and overall  
312 virulence. However, deficiency in site 4 does have a consistent but not always  
313 significantly greater effect upon these factors.

314

315 *Virus-cell interaction in the PLN influences replication in CNS*

316 As we have demonstrated above, reducing the number of miR-142-3p binding  
317 sites in the EEEV 3' UTR leads to increased virus attenuation *in vivo*. An important

318 question regarding the pathogenesis of arboviral encephalitic viruses is whether or not  
319 attenuation of encephalitis-causing viruses can be influenced by responses elicited in  
320 tissues outside the CNS. This is particularly significant with EEEV as we have proposed  
321 that failure to elicit a robust peripheral innate immune response contributes to the  
322 extreme virulence of the virus. Tissues from CD-1 mice infected with the EEEV mutants  
323 were harvested 96 hours post infection to measure virus replication in the PLN, spleen,  
324 and several regions of the brain. In the PLN and spleen, the triple, quadruple and 11337  
325 mutant viruses all had lower levels of mean virus replication compared to WT (S3  
326 Figure). These mutants also elicited higher levels of serum IFN- $\alpha/\beta$  than WT at 12 hpi.  
327 There was no difference in virus replication between WT and the single or double  
328 mutants at this time point suggesting that early IFN- $\alpha/\beta$  production by the triple,  
329 quadruple, and 11337 (Figure 3C), in the absence of complete miR-142-3p restriction,  
330 led to reduced virus replication *in situ* in the PLN and spleen.

331 We previously demonstrated that higher levels of serum IFN- $\alpha/\beta$  lead to  
332 increased STAT1 phosphorylation and, presumably, upregulation of the IFN-induced  
333 antiviral state in brain tissue at early times post EEEV infection prior to neuroinvasion by  
334 the virus [28]. At 96 hours post infection with the EEEV mutants, we harvested the  
335 cortex, subcortex, cerebellum and olfactory bulbs to measure virus replication by nLuc  
336 analysis. WT-infected mice exhibited high levels of virus replication in all brain regions  
337 (Figure 4A-D) corresponding to the time when WT EEEV infected mice begin to  
338 succumb to infection (Figure 3A). Similar levels of virus replication were also detected in  
339 the different regions of the brain infected with the single mutants and double mutants.

340 Even though all of the mice infected with the double mutant viruses had virus replication  
341 in the different regions of the brain, not all mice succumbed to infection (Figure 3A).

342 Virus replication was significantly lower for the triple mutants, the quadruple  
343 mutant 1234, and the deletion mutant 11337, compared to WT in all four regions of the  
344 brain. In fact, virus replication of these mutants was also lower than the single and  
345 double mutants at this time point. As further evidence of the contribution of extraneuronal  
346 replication to the attenuated phenotype of binding site mutants, we found that the WT  
347 and the most attenuated 11337 mutant viruses caused similar virulence when give  
348 intracerebrally to CD-1 mice (S4 Figure). These results demonstrate that the miR-142-  
349 3p binding sites not only suppress virus replication in the PLN early after infection, but  
350 the IFN- $\alpha/\beta$  induced early after infection reduces virus dissemination to the CNS  
351 contributing to the attenuation of these viruses *in vivo*.

352  
353 *Spontaneous mutation miR-142-3p binding sites may contribute to changing EEEV*  
354 *phenotypes as infection progresses.*

355 EEEV is primarily transmitted between ornithophilic mosquitoes and passerine  
356 birds, but infections do occur in humans and horses, both of which are considered  
357 dead-end hosts [29] and, therefore, should not contribute to the host-driven evolution of  
358 the virus. Mosquito cell lines do not express miR-142-3p [4,30] while avian  
359 hematopoietic cell lines do [31]; however, it may be expressed at lower levels than in  
360 mammal hematopoietic cell lines [4]. Importantly, without the miR-142-3p binding sites  
361 in the 3' UTR, EEEV cannot establish a productive infection in mosquitoes [4],  
362 suggesting that the miR-142-3p binding sites are maintained due to selective pressures

363 in the mosquito-bird life cycle but not during replication in humans or possibly mice as  
364 human disease models. Remarkably, the sequence of the region of the EEEV 3'UTR  
365 containing the miR-142-3p binding sites is identical between currently circulating viruses  
366 and viruses isolated in the 1930's, also suggesting a strong selective pressure for  
367 conservation [4]. Therefore, we sought to determine if some of the altering phenotype  
368 of EEEV during mouse infection, such as the increase in serum IFN- $\alpha\beta$  levels in mice  
369 infected with the WT or single mutation viruses between 12 and 24 hpi (Figure 3C-D),  
370 might be associated with a reduction in miR-142-3p restriction in myeloid cell infection.  
371 Interestingly, in RAW cells and interferon non-responsive mouse BMDCs, between 24  
372 and 48 hpi, WT EEEV appears to escape miR-142-3p suppression and replicates to  
373 high titers (Figure 5A-B).

374 To detect escape mutations *in vitro* and *in vivo*, RNA was isolated from WT  
375 EEEV-infected RAW cells (48 hpi), *Ifnar*<sup>-/-</sup> bone marrow derived dendritic cells (BMDCs)  
376 (24 hpi and 48 hpi), mouse brains, sera, and cervical lymph nodes (CVLN) at times >24  
377 hours post infection. PCR amplification of the 3' UTR led to the identification of a subset  
378 of amplified fragments in all amplified RNA that were smaller than the WT amplicon;  
379 however, with mouse samples, these fragments did not represent the majority of the  
380 viral population (data not shown). By comparison, in BMDCs, at 24 hpi a majority of the  
381 population was similar to WT (~750nt), but by 48 hpi, the predominant viral population  
382 had a much shorter 3' UTR more similar to 11337 (Figure 5C). In general, 3'UTR length  
383 variations were of multiple lengths (S5 Figure). However, amplicon sequencing revealed  
384 that in all circumstances, deletions encompassed most or all four of the miR-142-3p  
385 binding sites and the sequence of one sample from the serum of an infected mouse was

386 identical to the 11337 mutation (mutation 11337-11596). Two samples from the brains  
387 of B6 mice had identical deletions (11355-11601). These results demonstrate that the  
388 miR-142-3p binding sites are not stable during EEEV infection in a model of human  
389 disease.

390

391 *miR-142-3p binding sites are present in the EEEV-derived 3' UTR of western equine*  
392 *encephalitis virus.*

393 Western equine encephalitis virus (WEEV) is a recombinant alphavirus  
394 consisting of the 5' UTR, nonstructural proteins, capsid protein, and part of the 3' UTR  
395 of EEEV and the structural proteins (E3, E2, and E1) and the initial 60 nucleotides (nt)  
396 of the SINV 3' UTR [32,33]. Like EEEV, replication of WEEV is inhibited in human  
397 peripheral blood leukocytes[34]. Since part of the WEEV 3' UTR is derived from an  
398 EEEV progenitor, we hypothesized that the 3' UTR may also contain miR-142-3p  
399 binding sites. Screening of the WEEV 3' UTR of the McMillan strain (McM) identified  
400 four potential miR-142-3p binding sites located at different positions relative to the poly  
401 (A) tract and each other (Figure 6A-B) as compared to the miR-142-3p binding sites in  
402 the EEEV 3' UTR (Figure 1A). Two of the miR-142-3p binding sites, beginning at nt  
403 11347 and 11369, have canonical seed sequence matches, but they overlap in the 3'  
404 UTR by 6 nts. The other 2 miR-142-3p binding sites, beginning at 11405 and 11429,  
405 have a G:U wobble at position 2 of the seed sequence suggesting they may be non-  
406 canonical (Figure 6B).

407 To determine whether the miR-142-3p binding sites within the WEEV 3' UTR  
408 suppress WEEV replication in myeloid cells, we made a deletion mutant, WEEV-11224,

409 that deletes 226 nucleotides from the 3' UTR that eliminates all of the miR-142-3p  
410 binding sites similar to the 11337 deletion in EEEV[4]. In BHK cells, both WEEV McM  
411 and WEEV-11224 replicated with similar growth kinetics demonstrating that the deletion  
412 in the 3' UTR had no effect on virus replication in miR-142-3p deficient cells (Figure 6C)  
413 [4,30]. In RAW cells that express miR-142-3p [4], both WEEV McM and WT EEEV did  
414 not replicate (Figure 6D). Deletion of the miR-142-3p binding sites in McM (WEEV  
415 11224 virus) resulted in a 2-log increase in virus replication at 24 hpi compared to  
416 WEEV McM ( $P<0.01$ ). By comparison, EEEV 11337 replicated to a ~4-log higher titer  
417 than WEEV 11224 at 24 hpi. Differences between EEEV and WEEV in relative growth  
418 of WT viruses and miR-142-3p deletion mutants may reflect differential sensitivity to  
419 mutant-induced interferon responses or other myeloid cell factors as similar responses  
420 were seen in *Ifnar*<sup>-/-</sup> BMMφ (Figure 6E). Like EEEV 11337, WEEV 11224 is also  
421 inhibited in replicating in C6/36 mosquito cells (Figure 6F) suggesting a common  
422 function of this region between WEEV and EEEV in establishing mosquito replication.  
423 Finally, sc infection of mice revealed that the 11224 mutant was attenuated ( $P=0.052$ )  
424 versus the WT WEEV McM (25% versus 75% mortality, respectively) (Figure 6G).  
425 Attenuation of the EEEV miR-142-3p deletion mutant 11337 is due to differential type I  
426 IFN induction[4], therefore we infected *Ifnar*<sup>-/-</sup> mice to determine whether WEEV 11224  
427 was similarly attenuated by the type I IFN response. Survival times and mortality of  
428 *Ifnar*<sup>-/-</sup> mice infected with WEEV McM and WEEV 11224 were not distinguishable  
429 (Figure 6G) demonstrating that type I IFN is needed for the attenuation of WEEV 11224  
430 compared to WEEV McM. Thus, similar to EEEV, the miR-142-3p binding sites in the

431 WEEV McM 3' UTR restrict virus replication in myeloid cells leading to suppressed IFN  
432 responses and increased virulence in mice.

433 **DISCUSSION**

434 miRNAs function in a cell and tissue specific manner to prevent translation of  
435 cellular mRNAs. Recent evidence demonstrates that cellular miRNAs can interact with  
436 RNA viruses to either suppress RNA translation via interaction with 3' UTRs or enhance  
437 virus replication by interacting with 5' UTRs (reviewed in [19]). Artificial insertion of  
438 miRNA binding sites into viral 3' UTRs has been used to restrict virus replication in  
439 specific cells or tissues to understand the contribution of these cells and tissues to viral  
440 pathogenesis. These experiments require insertion of multiple highly complementary  
441 miRNA sequences in specific locations and distances from each other to achieve  
442 efficient viral suppression [22,23,30,35,36]. However, miRNA natural binding sites in  
443 viral 3' UTRs are not completely complimentary nor are they arranged at uniform  
444 distances from each other [4]. We have used point mutations to determine the role of  
445 each of four naturally occurring canonical and non-canonical miR-142-3p sites whose  
446 stringent restriction of myeloid cell replication drives the severity of encephalitis caused  
447 by EEEV, one of the most acutely virulent viruses endemic to the Americas. Through  
448 these studies, we have confirmed that miR-142-3p interacts with the EEEV 3' UTR as  
449 part of the RISC complex in myeloid cells *in vitro* and we have determined that all four  
450 of the miR142-3p sites, including the non-canonical site 2, contribute to EEEV virulence.  
451 Furthermore, we demonstrate that another encephalitic alphavirus, WEEV, achieves  
452 virulence through miR-142-3p restriction.

453 Viruses [22] and cellular mRNA [37-39] encoding multiple miRNA binding site are  
454 more suppressed than viruses and mRNA encoding only a single miRNA binding site.  
455 This interaction between two or more miRNA binding sites leading to enhanced

456 repression of the target RNA over a single miRNA binding site is called cooperativity  
457 [38-40]. Cooperativity between miRNAs is most optimal when the seed sequences of  
458 the miRNA binding sites are spaced 13-35 nucleotides apart [40]. Also, the location of a  
459 miRNA binding site in the 3' UTR within 15 nucleotides from the stop codon can  
460 increase efficacy and repression of the target RNA [38]. However, not all naturally  
461 encoded viral miRNA interaction domains encode more than one miRNA binding site  
462 [17,18,41,42], and if there is more than one miRNA binding site, the sites may not be  
463 ideally located based on the aforementioned guidelines for cooperativity.

464 For EEEV, the number of miR-142-3p binding sites rather than their location  
465 within the 3' UTR was dominant in the suppression of EEEV replication. EEEV mutants  
466 with 2 or more miR-142-3p binding sites were suppressed in replication in myeloid cells  
467 *in vitro* to WT EEEV and *in vivo* in the PLN. Once a third miR-142-3p binding site was  
468 removed from the 3' UTR leaving only one intact miR-142-3p binding site, EEEV virus  
469 replication was rescued in myeloid cells and the PLN. Even though replication was  
470 detected with the triple mutant viruses, it was still lower than the quadruple miR-142-3p  
471 mutant, 1234, and the deletion mutant, 11337 indicating that each miR-142-3p binding  
472 site contributes to EEEV restriction *in vitro*. However, it should be noted that mutant 4  
473 exhibited a non-significant but consistent trend towards higher replication potentially  
474 implying a more substantial contribution to EEEV restriction that the other binding sites.

475 With *in vivo* morbidity/mortality studies, which appeared to distinguish more  
476 precisely between effects of individual binding sites, some of the single, double, and  
477 triple mutants were significantly attenuated in comparison with WT EEEV. Of the single  
478 mutants, the mutant EEEV virus lacking site 4 was the most attenuated. This miR-142-

479 3p binding site is located the closest to the poly (A) tail, a known factor in enhancing  
480 miRNA restriction of cellular mRNA [38], and its position within the UTR may underlie  
481 greater replication restriction observed *in vitro* and *in vivo*. The mutant virus 34, which  
482 was mutated in two sites that are only separated by 8 nucleotides, was the most  
483 attenuated double mutant virus compared to WT EEEV. This suggests that there is  
484 some positional effect for these sites that may enhance cooperativity. Of the triple  
485 mutants, the viruses mutated in both sites 3 and 4 in conjunction with either site 1 or 2  
486 were the most attenuated, again suggesting greater contribution of site 4 and an  
487 interaction between sites 3 and 4.

488 All of the triple mutants were attenuated, but more virulent than the quadruple  
489 mutant 1234 or 11337. Site two with a G:U wobble at position 2 of the seed sequence is  
490 a non-canonical miRNA binding site due to a potential offset 6-mer binding site that has  
491 complementarity between nucleotides 3-8 of the seed sequence and the EEEV 3' UTR  
492 [43]. The nullification of this non-canonical site in the mutant 134 still resulted in  
493 increased mortality during infection compared to 1234 and 11337. Therefore, our data  
494 demonstrate that each miR-142-3p binding site, even the non-canonical site 2, is  
495 involved in suppression of virus replication in myeloid cells and attenuation of EEEV  
496 disease. However, as noted, site 4 may be the most restrictive by itself and positional  
497 effects between sites 3 and 4 may enhance cooperativity yielding the greater  
498 suppression of replication.

499 It can be argued that the binding of miRNAs to viral RNAs can lead to  
500 sequestration of the miRNA leading changes in the host transcriptome due to  
501 derepression of previously repressed targets [13,44] and with EEEV, potentially causing

502 altered myeloid cell responses to infection. However, the binding of miRNAs to viral  
503 RNA is unlikely to result in changes in the cellular transcriptome through sequestration  
504 of the free miRNA early after infection when viral genomes are limited. We previously  
505 demonstrated with EEEV, that initial translation of the infecting virus genome is  
506 restricted by miR-142-3p and subsequent replication is greatly suppressed [4]. Here we  
507 show that EEEV RNA associates with Ago complexes and therefore, is directed into  
508 translation repression pathways. Furthermore if the miRNA is highly expressed within  
509 the cell, as with miR-142-3p in myeloid cells [3], the number of miR-142-3p molecules  
510 will greatly exceed EEEV RNA molecules particularly early after infection. The loss of a  
511 small number of miR-142-3p molecules due EEEV sequestration should not  
512 quantitatively change the levels of miR-142-3p within myeloid cells.

513 Our results also point to an effect of replication efficiency in initial infection sites  
514 on subsequent CNS disease severity. IFN- $\alpha/\beta$  production by the triple, quadruple and  
515 11337 mutants at 12 hpi resulted in reduced virus dissemination to the CNS (Figure 5)  
516 suggesting that the initial innate immune responses within 12 hours after infection can  
517 influence events in tissues distant form the site of infection. We have previously shown  
518 production of serum IFN can lead to an upregulation of STAT1 transcription factor  
519 phosphorylation in the CNS [28]. Furthermore, WT EEEV and the 11337 mutant,  
520 missing all four sites, were equally virulent when virus was delivered ic. Therefore, the  
521 early IFN response in the triple, quadruple and 11337 deletion mutants may help to  
522 prime the innate immune response in the CNS to restrict virus replication and lessen  
523 disease severity. Myeloid cell production of cytokines and chemokines are also integral  
524 to the induction of both the innate and adaptive immune responses *in vivo* [26,27]. The

525 inability of EEEV to replicate in tissue-specific myeloid cells may also lead to  
526 suppression of the cytokine and chemokine leading to inadequate induction of the  
527 adaptive immune responses in the spleen, or recruitment of important cell types to the  
528 CNS needed for neuroprotection. We infer that limitations in neuroprotective adaptive  
529 immune responses may also contribute to the extremely high mortality rate in  
530 symptomatic EEEV cases.

531 There is remarkable conservation of the EEEV 3' UTRs that have been isolated  
532 from nature with a majority of the EEEV strains having nearly identical sequence and  
533 location of the miR-142-3p binding sites as the prototype strain EEEV FL93-939 [4]. Our  
534 data suggests that requirements for mosquito cell replication exert a strong selective  
535 pressure for maintenance of the miR-142-3p sites (current studies and [4]), which may  
536 explain such conservation. However, we found that in mammalian cells and hosts, the  
537 lack of a selective pressure results in generation of escape mutants that lack the miR-  
538 142-3p binding sites. Similarly, artificial miR-142-3p binding sites introduced into  
539 Dengue virus were deleted during the course of mouse infection [30]. The EEEV  
540 mutations we observed rendered all four of the miR-142-3p sites non-functional  
541 reinforcing the contribution of all sites to EEEV replication restriction.

542 Finally, we have shown that WEEV, a natural recombinant SINV/EEEV virus that  
543 derived its 3' UTR sequences from an EEEV-like ancestor, contains four binding sites  
544 for miR-142-3p (Figure 6), but in different locations in the 3' UTR compared to EEEV.  
545 Deletion of the binding sites from WEEV rescued virus replication in myeloid cells and  
546 attenuated the mutant virus in mice, while also suppressing replication in mosquito cells.  
547 Therefore, we suggest that multiple members of the encephalitic alphaviruses express

548 virulence factors conferred by miRNA binding. It is of interest that the other major  
549 encephalitic alphavirus, Venezuelan equine encephalitis virus, has a much shorter 3'  
550 UTR than EEEV or WEEV [45], does not possess miR-142-3p binding sites and is  
551 highly myeloid cell tropic [2].

552 In summary, our data demonstrate that the miR-142-3p binding sites  
553 cooperatively suppress EEEV 3' UTR replication in myeloid cells thereby enhancing  
554 virulence *in vivo*. miRNA binding sites are being used to limit tissue tropism in the  
555 generation of live-attenuated vaccine candidates [22,23]. However, these artificial  
556 miRNA binding sites are usually complimentary to the entire miRNA [22,23,30], which is  
557 rarely found in naturally occurring viral RNAs [4,13-17]. Furthermore, when artificial  
558 miRNA binding sites are grouped together, viral escape of miRNA suppression can  
559 occur through deletion of the inserted miRNA binding sites [22,30], leading to potential  
560 adverse events.

561 **Materials and Methods**

562 **Ethics Statement**

563 All animal procedures were carried out under approval of the Institutional Animal Care  
564 and Use Committee of the University of Pittsburgh in protocols 15066059 and  
565 18073259. Animal care and use were performed in accordance with the  
566 recommendations in the Guide for the Care and Use of Laboratory Animals of the  
567 National Research Council. Approved euthanasia criteria were based on weight loss  
568 and morbidity.

569

570 **Cell culture:** Baby hamster kidney cells (BHK-21), L929 fibroblasts, RAW 264.7 (RAW)  
571 monocyte-macrophage cells, and *Aedes albopictus* C6/36 mosquito cells were  
572 maintained as previously described[2,4]. Bone marrow-derived, conventional dendritic  
573 cells were generated from C57BL/6 mice (Jackson Laboratories) and maintained as  
574 previously described[2] in media supplemented with 10ng/ml recombinant interleukin-4  
575 and 10ng/ml granulocyte-macrophage colony stimulating factor (Peprotech). Bone  
576 marrow macrophages (BMMΦ) were generated from *Ifnar*<sup>-/-</sup> mice as previously  
577 described [2] in Dulbeccos' modified Eagle's medium supplemented with 20% L929-  
578 conditioned supernatant.

579

580 **Viruses:** Construction of the WT EEEV strain FL93-939 cDNA clone and mutant 11337  
581 cDNA clone encoding nanoLuciferase (nLuc) as a cleavable in-frame fusion protein  
582 located between the capsid and E3 protein has been described previously [4,24,46].  
583 EEEV mutant viruses containing three nucleotide mutations in the miR-142-3p binding

584 sites in the 3' UTR complimentary to the miR-142-3p seed sequence to eliminate miR-  
585 142-3p binding were generated singly or in combination using the QuikChange  
586 Mutagenesis II XL kit and the primers listed in S1 Table [4]. The infectious cDNA clone  
587 of WEEV McMillan strain (McM) was kindly provided by Kenneth Olson, Colorado State  
588 University[47]. This clone was modified by placing the entire virus sequence into the  
589 PBR-322 based vector of the FL93-939 virus cDNA under transcriptional control of the  
590 T7 bacteriophage promoter. A WEEV McM miR-142-3p mutant virus (WEEV 11224)  
591 was created by deleting 226 nucleotides in the 3' UTR (nucleotide 11224 to 11449) by  
592 QuikChange Mutagenesis using the primers listed in S1 Table. Capped, *in vitro*  
593 transcribed RNA was generated from the linearized cDNA using the T7 mMessage  
594 mMachine kit (Ambion), and electroporated into BHK cells as previously described [2].  
595 Titers of virus stocks was determined by BHK-21 cell plaque assay.

596

597 **Translation Reporters and RNA immunoprecipitations:** Generation of the WT  
598 EEEV, and 11337 translation reporters were described previously [4]. Capped, *in vitro*  
599 transcribed RNA was generated for use in immunoprecipitation assays. Raw cells were  
600 transfected with 7ug reporter RNAs either with or without 30 pmoles of biotinylated miR-  
601 142-3p mimic or scrambled 3' biotinylated mimic (Dharmacon) using the Neon  
602 Transfection System (Invitrogen; 1750V, 25ms, 1 pulse). At 1.5-2h post-transfection,  
603 cells were washed thrice using ice-cold PBS w/o Ca<sup>+</sup> and Mg<sup>+</sup>. Lysates were collected  
604 on ice in modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.4,  
605 150 mM NaCl, 1% NP-40) supplemented with protease inhibitors (1 mM  
606 phenylmethylsulfonyl fluoride, 1μg/ml leupeptin, and 1 μg/ml pepstatin), and a

607 phosphatase inhibitor cocktail (Sigma). Lysates were spun at 12000g for 10 min at 4°C  
608 to clear debris and supernatants transferred to pre-chilled tubes. For  
609 immunoprecipitation of the Argonaute complex, Protein A/G Plus agarose beads were  
610 blocked with bovine t-RNA (1mg/mL; Sigma) for 2h in modified-RIPA buffer, washed 2X  
611 and resuspended in modified-RIPA buffer. Lysates were pre-cleared by adding 1µL  
612 rabbit serum and 30µL Protein A/G Plus agarose beads (30 µL per sample; Santa Cruz)  
613 for 2h at 4°C on a nutator. Lysates were spun at 2500g for 10 min at 4°C and  
614 supernatants transferred to pre-chilled tubes. Anti-eiF2C rabbit polyclonal antibody (30  
615 µL per sample; Santa Cruz; H-300) was added and rocked on a nutator O/N at 4°C. t-  
616 RNA blocked protein A/G beads were added for an additional 2h at 4°C. Lysates were  
617 spun at 2500g and washed 3X in ice-cold RIPA buffer. Beads were suspended in Trizol  
618 reagent (Ambion) and freeze-thawed at -80°C. For immunoprecipitation of the  
619 biotinylated mimic RNA, streptavidin agarose beads (30µL per sample; Cell Signaling)  
620 were blocked with bovine t-RNA (1mg/mL; Sigma) for 2h in RIPA buffer, washed 2X and  
621 resuspended in RIPA buffer. t-RNA blocked streptavidin beads were added for 6h at  
622 4°C on a nutator. Lysates were spun at 2500g and washed 3X in ice cold RIPA buffer.  
623 Beads were suspended in Trizol reagent (Ambion) and freeze-thawed at -80°C. For  
624 both immunoprecipitations, RNA was extracted using manufacturers guidelines  
625 (Ambion). 100 ng of total RNA was used in a reverse transcription reaction with random  
626 hexamer (Integrated DNA Technologies), and resultant cDNA was used to detect levels  
627 of reporter RNAs with the following primers: sense (5'-  
628 GGGAGCGCGCCTGTAAGGCACAC-3') and antisense (5'-

629 GCTCTCCAGCGGTTCCATCTTCCAGC-3'). Data was normalized to mock samples  
630 and total input RNA levels.

631

632 **Virus Growth Curves:** BHK (2x10<sup>5</sup> cells/well), RAW (2x10<sup>5</sup> cells/well), BMDCs  
633 (1.5x10<sup>5</sup> cells/well), and C6/36 cells (2x10<sup>5</sup> cells/well) were seeded in 24 well plates one  
634 day prior to infection. Viruses were infected in triplicate at a MOI = 0.1 for RAW and  
635 BHK cells, MOI = 5 for BMDCs, or MOI = 1 for C6/36 cells in phosphate buffered saline  
636 (PBS) supplemented with 1% FBS. After 1 hour, the cells were washed with PBS and  
637 complete media was added to each well. For nLuc analysis, at the indicated time points,  
638 the cells were washed three times with PBS followed by addition of 100ul of 1X Passive  
639 lysis buffer (PLB, Promega). The cells were then scraped and transferred to a 96 well  
640 plate. For plaque assay, supernatant was collected at time zero and indicated time  
641 points for titration by plaque assay on BHK-21 cells.

642

643 **Mouse infection and tissue collection:** 6-week old female outbred CD-1 mice  
644 (Charles River Laboratories) or C57BL/6J (Jackson Laboratories) were infected  
645 subcutaneously (sc) in each footpad with 10<sup>3</sup> pfu of nLuc-expressing EEEV mutant  
646 viruses in 10 ul of OptiMEM media (Invitrogen). For WEEV, female *Ifnar*<sup>-/-</sup> and CD-1  
647 mice were infected with 10<sup>4</sup> pfu of WEEV McM or WEEV 11224 sc in each footpad. All  
648 mice were scored daily for clinical signs and weight loss as described previously [2]. For  
649 tissue collection, serum was collected via the submandibular vein, and the mice were  
650 perfused with PBS. Tissues were harvested and collected into Eppendorf tubes  
651 containing 1x PLB (e.g.100ul per a single popliteal lymph node (PLN), 400ul per

652 footpad, 800ul per spleen and brain), or Tri-Reagent for RNA analysis. For aerosol  
653 infection, CD-1 mice were challenged with 100LD<sub>50</sub> of EEEV FL93 as previously  
654 described [25]. On day 5 post infection, cervical lymph nodes (CVLN) were harvested  
655 and processed in Tri-Reagent prior to being frozen at -80°C. Samples in PLB were  
656 homogenized and refrozen at -80°C prior to analysis. All animal procedures were  
657 carried out in accordance with the American Association for the Accreditation of  
658 Laboratory Animal Care International-approved institutional guidelines for animal use  
659 and approved by the University of Pittsburgh Institutional Animal Care and Use  
660 Committee.

661

## 662 **Luciferase Assays and Protein Assays**

663 nLuc assays were using the Nano-Glo Luciferase system (Promega) and preformed  
664 according to manufacturer's guidelines. Samples were diluted in 1x PLB for  
665 determination of nLuc relative light units (RLU) using a Orion microplate luminometer  
666 (Berthold) or a FLUOstar Omega microplate reader (BMG Labtech). RLU was  
667 normalized to protein levels in samples determined by a bicinchoninic acid protein  
668 assay (Pierce).

669

## 670 **Interferon (IFN- $\alpha/\beta$ ) Bioassays**

671 Biologically active serum IFN- $\alpha/\beta$  collected at 12 and 24 hpi was measured using a  
672 standard IFN biological assay on L929 cells as described previously [48]. The IFN- $\alpha/\beta$   
673 concentration in sera samples was set as the dilution of sample required for 50%

674 protection from cytopathic effect compared to protection conferred by an IFN standard  
675 [25].

676

### 677 **RNA Isolation and RT-PCR**

678 RNA was isolated from PLN in Tri-reagent according to manufacturer's guidelines. Poly-  
679 acryl carrier was added to each PLN prior to addition of 1-bromo-3-chloropropane (BCP)  
680 and phase separation. Reverse transcription (RT) of 100 ng of RNA was performed as  
681 previously described [49] using Moloney Murine Leukemia virus (M-MLV) reverse  
682 transcriptase (Promega), with an extension temperature of 42°C for 60 min and random  
683 hexamer (IDT). For cytokine and chemokine analysis, Maxima qPCR SYBR Green/ROX  
684 Master Mix (ThermoFisher) was used and the primers in S2 Table. Threshold cycle ( $C_T$ )  
685 values were normalized to 18s and compared to mock samples using the  $\Delta\Delta C_T$  method.

686

### 687 **Identification of EEEV Escape Mutants**

688 RNA was isolated as described from *in vitro* cultured RAW cells or BMDCs at 48 hr post  
689 infection or EEEV-infected (1x10<sup>3</sup> pfu bilaterally in footpad) and brain (D5), serum (24  
690 hpi), and CVLN (D5) samples were harvested and placed in Tri-Reagent for RNA  
691 analysis. RT was performed using 50uM Oligo(dT) (Thermo Fisher) as previously  
692 described [49] with an 48°C extension temperature. cDNA was diluted with H<sub>2</sub>O and 10ul  
693 was used in a GoTaq PCR reaction (GoTaq Green Mastermix, Fisher Scientific) with the  
694 following conditions: 95°C 2 min, (95°C 45s, 60°C 30s, 73°C 60s) x 40 cycles, 73°C 7  
695 min. The following primers were used: EEEV 10951-S:  
696 CGTTGCCTACAAATCCAGTAAAGCAGGA; T7-EEECSE-AS:

697 TAATACGACTCACTATAGGGCGTATGGAAAAAATTAATATGATTTGTAAATTGATAT  
698 AAAAGACAGC. The entire PCR reaction was run on a 2% agarose TAE gel followed  
699 by excising the bands and clean-up using Wizard SV Gel Clean-up (Promega). cDNA  
700 from WT EEEV and 11337 stocks were used as positive controls during PCR.  
701 Sequencing was performed by the University of Pittsburgh HSCRF Genomics Research  
702 Core and analyzed using CLC Genomics Workbench (Qiagen).

703

704 **Software and Statistical Analysis**

705 miRANDA-3.3a software [50,51] was used to align the mmu-miR-142-3p sequence with  
706 the EEEV FL93 genome (EF151502.1) and WEEV McMillan genome (GQ287640.1). All  
707 statistical analysis was performed using GraphPad Prism software. All experiments  
708 were repeated at least twice as indicated in Figure Legends. For IP, unpaired t test was  
709 performed to compared between groups. For the EEEV point mutant *in vitro* and *in vivo*  
710 data, a one-way analysis of variance was performed of the log-transformed data with  
711 corrections for multiple comparison using the Holm-Sidak method comparing each  
712 mutant to WT. An unpaired t-test was used to compare 11337 to mutant 1234 in the  
713 growth curve experiments in C6/36 cells. Box-and whisker plots represent min-max with  
714 bar representing the median value. Statistical significance for survival curves was  
715 determined by Mantel-Cox log rank test compared to WT. For the WEEV growth curves,  
716 multiple unpaired t tests were perfumed and corrected for multiple comparisons using  
717 the Holm-Sidak method.

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885

886 **Figure Legends**

887 **Figure 1: miR-142-3p binds to EEEV 3' UTR in myeloid cells.** A) EEEV genome  
888 structure. miR-142-3p binds (red bar) to EEEV genome in the 3' UTR. Numbers indicate  
889 nucleotide position in the EEEV genome of the 3' UTR and miR-142-3p binding sites B)  
890 EEEV or EEEV 11337 translation reporters (7ug) were electroporated into the RAW  
891 monocyte/macrophage cell line. Anti-Ago1/2 antibody was used to immunoprecipitation  
892 of Argonaute proteins. qRT-PCR was used to quantify amount of reporter RNA in each  
893 immunoprecipitated fraction. Data is represented as fold change over mock. \*\*P<0.01,  
894 NS: not significant unpaired t test . n=2 independent experiments. Error bars represent  
895 SD. C) Biotin labeled miR-142-3p mimic or biotin labeled scramble (sc) were co-  
896 electroporated with the reporters into RAW cells. Streptavidin beads was used for  
897 immunoprecipitation and qRT-PCR was to quantify the amount of reporter RNA in each  
898 fraction. Data is represented as fold change over sc. n=2-3 independent experiments.  
899 Error bars represent SD. \*\*\*P<0.001, unpaired t test. D) Alignment of EEEV '3 UTR to  
900 mmu-miR-142-3p with red letter indicate mutated nucleotides (nt) in seed sequence. nt  
901 number indicates initial position in EEEV genome of miR-142-3p binding sites.

902

903 **Figure 2: Decreasing the number of miR-142-3p binding sites leads to increased**  
904 **virus replication in myeloid cells and viral attenuation *in vivo*.** A-C) Quantification  
905 of virus replication at 12 hpi. BHK (MOI=0.1), RAW (MOI=0.1), BMDCs (MOI=5) were  
906 infected with the EEEV mutants expressing nLuc. D-E) Virus replication in C6/36  
907 mosquito cells (MOI=1) at (D) 8hpi and (E) 12hpi. Data is  $\log_{10}$  transformed and  
908 expressed as relative light units (RLU) per  $\mu\text{g}$  protein. N=6-9, 2-3 independent

909 experiments. \*P<0.5, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 one way ANOVA with  
910 corrections for multiple comparisons using the Holm-Sidak method comparing each  
911 mutant to WT. Error bars represent SE.

912

913 **Figure 3: Elimination of miR-142-3p binding sites leads to attenuation due to**  
914 **increased virus replication in popliteal lymph node and serum IFN.** A) Female  
915 outbred CD-1 mice (5-6 weeks) were infected with  $10^3$  pfu sc in each footpad. Morbidity  
916 and mortality were measured twice daily. n=10 mice from 2 independent experiments  
917 \*P<0.5, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001, Log-Rank Test comparing each EEEV  
918 mutant to WT. B) Outbred CD-1 mice were infected with  $10^3$  pfu of WT, 11337 or each  
919 mutant sc in each footpad. A single popliteal lymph node (PLN) was harvested at 12 hpi  
920 and analyzed for nLuc expression. Data is  $\log_{10}$  transformed and represented as RLU  
921 per LN. n = 7-12 mice, from 2-3 experiments. Box-and whisker plots represent min-max  
922 with bar representing the median value. C-D) Biologically active IFN- $\alpha/\beta$  levels in serum  
923 at 12 hpi (B) and 24 hpi (C). CD-1 mice were infected with  $10^3$  pfu of WT, 11337 or each  
924 mutant sc in each footpad. n=8-12 mice, from 2-3 experiments. \*P<0.05, \*\*P<0.01,  
925 \*\*\*P<0.001, \*\*\*\*P<0.0001, comparing the log-transformed data of each mutant with WT  
926 using the one way analysis of variance test with corrections for multiple comparisons  
927 using the Holm-Sidak method. LOD = limit of detection of the IFN assay. Bar represents  
928 geometric mean.

929

930 **Figure 4: Reduced virus replication in the central nervous system after infection**  
931 **with the triple and quadruple mutant EEEV viruses.** CD-1 mice were infected with

932 10<sup>3</sup> pfu of the EEEV mutants sc in each footpad. Tissues were harvested at 96 hours  
933 post infection. Virus replication in cortex (A), subcortex (B), cerebellum (C), and  
934 olfactory bulb (D). Data is log<sub>10</sub> transformed and represented as either RLU/LN or RLU  
935 per µg protein N=8 mice, from 2 independent experiments. \*P<0.5, \*\*P<0.01,  
936 \*\*\*P<0.001, \*\*\*\*P<0.0001 one way analysis of variance test with corrections for multiple  
937 comparisons using the Holm-Sidak method comparing each mutant to WT. Box-and  
938 whisker plots represent min-max with bar representing the median value.

939

940 **Figure 5: Escape of miR-142-3p suppression in myeloid cells.** A-B) Replication of  
941 EEEV increases between 24 and 48 hpi *in vitro* detected by plaque assay in A) RAW  
942 cells or semi-quantitative RT-PCR in (B) *Ifnar*<sup>-/-</sup> BMDCs. n=6-9 individuals wells from 2-3  
943 independent experiments. Fold difference in B is compared to mock samples. Bar  
944 represents geometric mean and geometric SD. C) Representative gel from PCR of  
945 EEEV 3' UTR of BMDC escape mutant between 24 hpi and 48 hpi. Control PCR  
946 product is approximately 750nt for WT EEEV and 500nt for 11337. 1kb and 100bp  
947 indicate 1kb and 100bp ladders respectively.

948

949 **Figure 6: WEEV McMillan (McM) encodes miR-142-3p binding sites that restrict**  
950 **myeloid cell replication.** A) Alignment in genome and location of miR-142-3p binding  
951 sites in WEEV McM 3' UTR. B) Alignments between murine mmu-miR-142-3p and  
952 WEEV 3'UTR. C-F) Viral growth curves in BHK (C), RAW (D), *Ifnar*<sup>-/-</sup> BMMΦ (E), or  
953 C6/36 (F) of wild-type viruses (filled) or miR-142-3p mutants (open). Multiplicities of  
954 infection: BHK and RAW MOI=1, *Ifnar*<sup>-/-</sup> BMMΦ MOI=5. n=6 from 2 independent

955 experiments. Data was  $\log_{10}$  transformed and and multiple unpaired *t*-tests were  
956 performed and corrected for multiple comparisons using the Holm-Sidak method.  
957 \*p<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001. ++++P<0.0001 comparing WEEV McM  
958 versus WEEV 11224 in C6-36 cells. Data is represented as geometric mean with  
959 geometric SD. G) Infection of outbred CD-1 mice (n=10 mice, 2 independent  
960 experiments) or *Ifnar*<sup>-/-</sup> mice (n=13 mice, 2 independent experiments) with 10<sup>4</sup> pfu of  
961 wild-type WEEV McM or mutant WEEV 11224 sc in both rear footpads. Morbidity and  
962 mortality were monitored twice daily. Mantel-Cox Log rank test was used to compare  
963 between viruses.

964

965 **Supporting Information Captions**

966 **S1 Figure. EEEV mutants lacking functional miR-142-3p binding sites are**  
967 **attenuated in C57BL6 mice.** Female C57BL6 mice (5-6 weeks) were infected with  $10^3$   
968 pfu sc in each footpad. Morbidity and mortality were measured twice daily. n=7-8 mice  
969 from 2 independent experiments

970

971 **S2 Figure: Increased myeloid cell replication leads to increased cytokine and**  
972 **chemokine mRNA in PLN.** A) Cytokine and chemokine mRNA levels in the PLN of CD-  
973 1 mice 12 hpi with  $10^3$  pfu of WT, 11337 or mock infected. Data is represented as fold  
974 difference compared to mock mice. n=12 mice, 3 independent experiments B) *Cxcl10*  
975 mRNA levels in PLN 12 hpi with EEEV mutants. n=8-12 mice, 2-3 independent  
976 experiments \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001, (A) one way analysis of  
977 variance test with corrections for multiple comparisons using Turkey method or (B) one  
978 way analysis of variance test between WT and each mutant with corrections for multiple  
979 comparisons using Holm-Sidak method of the log-transformed data. ns=non-significant.  
980 Box-and whisker plots represent min-max with bar representing the median value.

981

982 **S3 Figure: Reduced virus replication in the periphery with the triple and**  
983 **quadruple mutant EEEV viruses.** CD-1 mice were infected with  $10^3$  pfu of the EEEV  
984 mutants sc in each footpad. Tissues were harvested at 96 hours post infection. Virus  
985 replication in PLN (A), spleen (B). N=8 mice, from 2 independent experiments. \*P<0.5,  
986 \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 one way analysis of variance test with corrections

987 for multiple comparisons using the Holm-Sidak method comparing each mutant to WT.

988 Box-and whisker plots represent min-max with bar representing the median value.

989

990 **S4 Figure: Mutant 11337 is virulent after intracerebral infection.** Survival of female

991 (5-6 week) CD-1 infected with ic with either  $10^3$  pfu of WT or 11337 mutant. Morbidity

992 and mortality were measured twice daily. n=8 mice from 2 independent experiments.

993

994 **S5 Figure: Escape mutants generated during infection eliminate miR-142-3p**

995 **binding sites in EEEV 3' UTR.** Alignment of escape mutants isolated for indicated cells

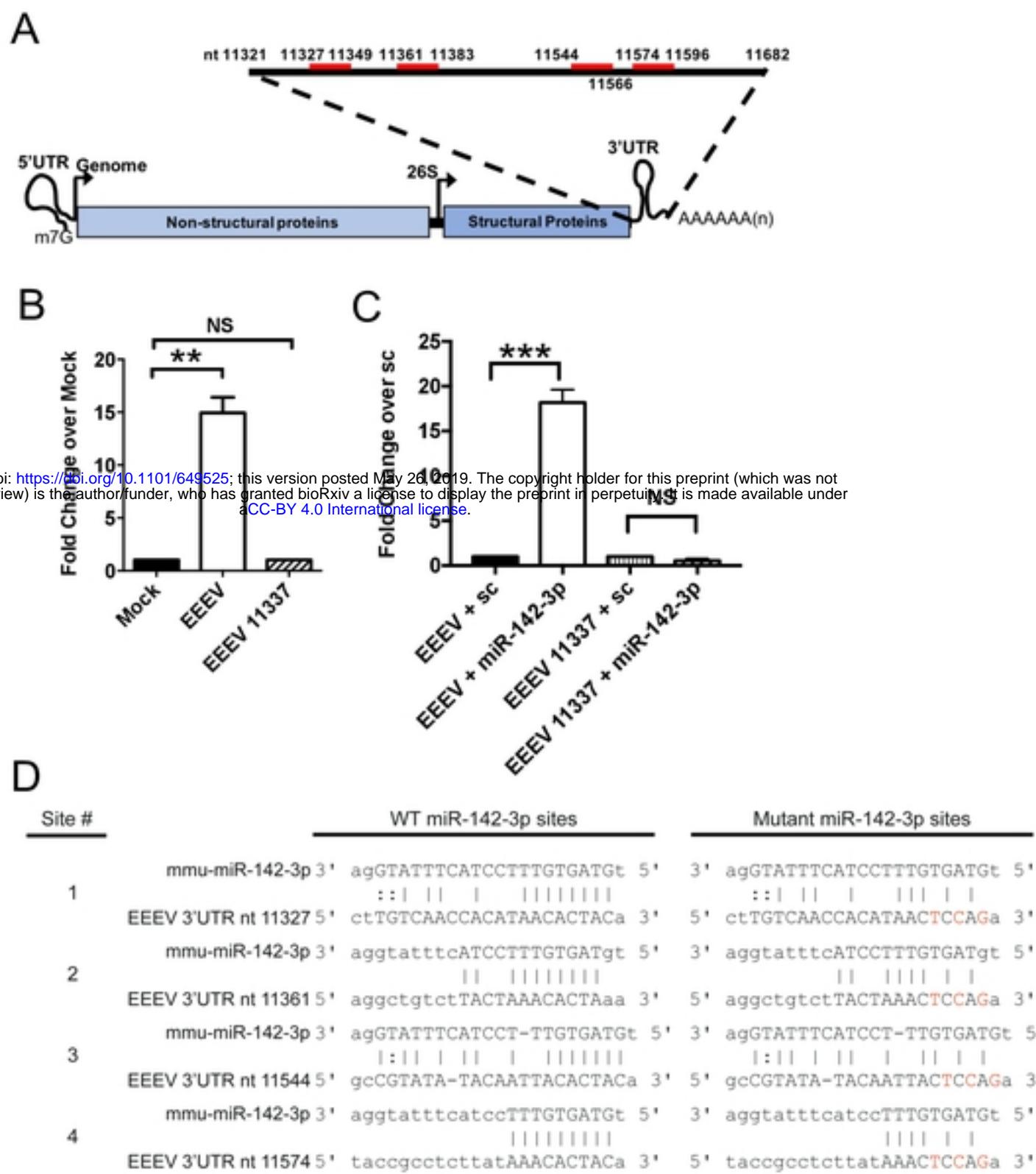
996 or tissues. Numbers on left indicate location in the genome of the deletion. Numbers at

997 end of the sequence indicate the length of the 3' UTR in each escape mutant. B6 -

998 C57Bl6, BMDC- bone marrow derived dendritic cell, CVLN – cervical lymph nodes

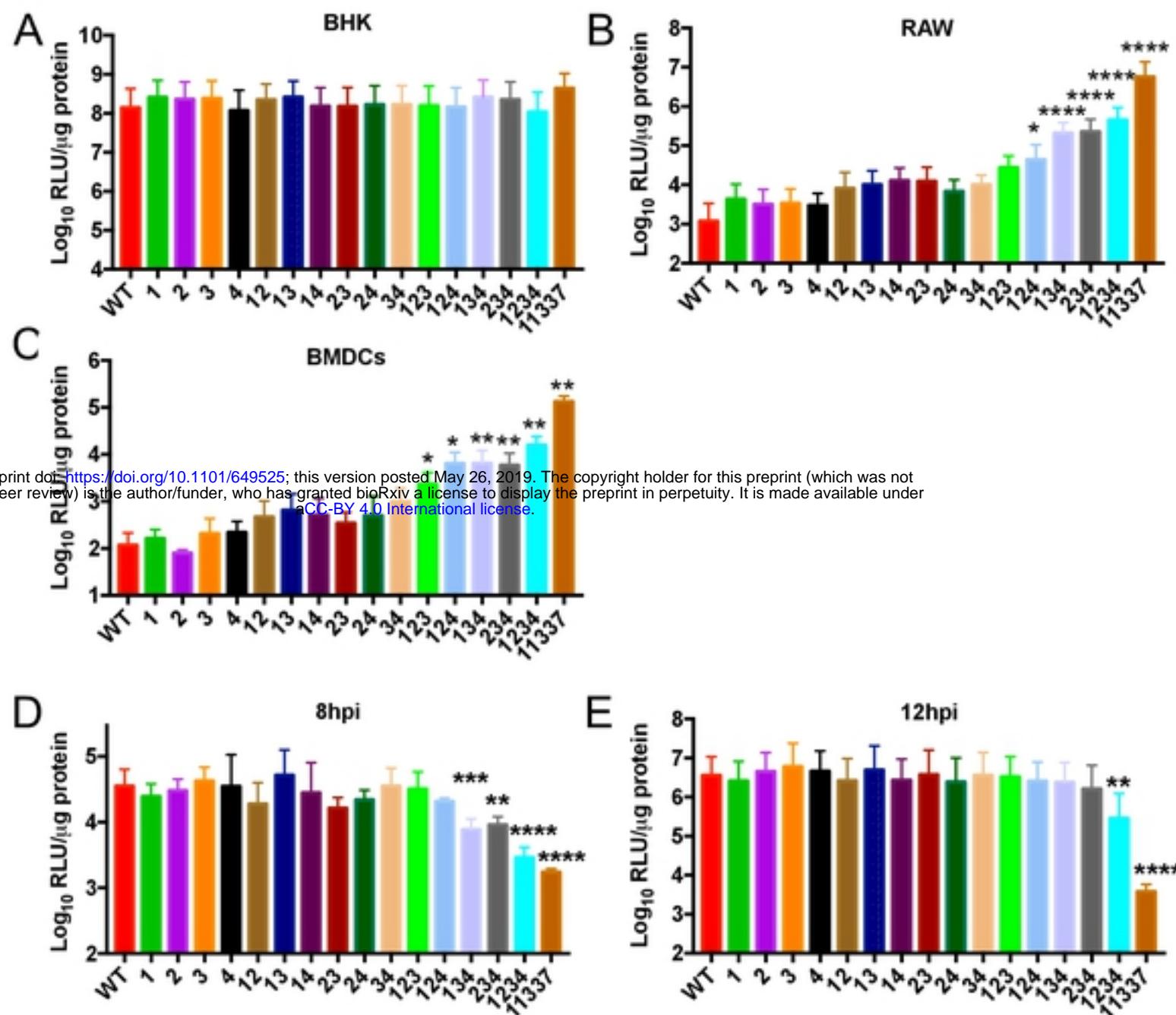
999

Figure 1



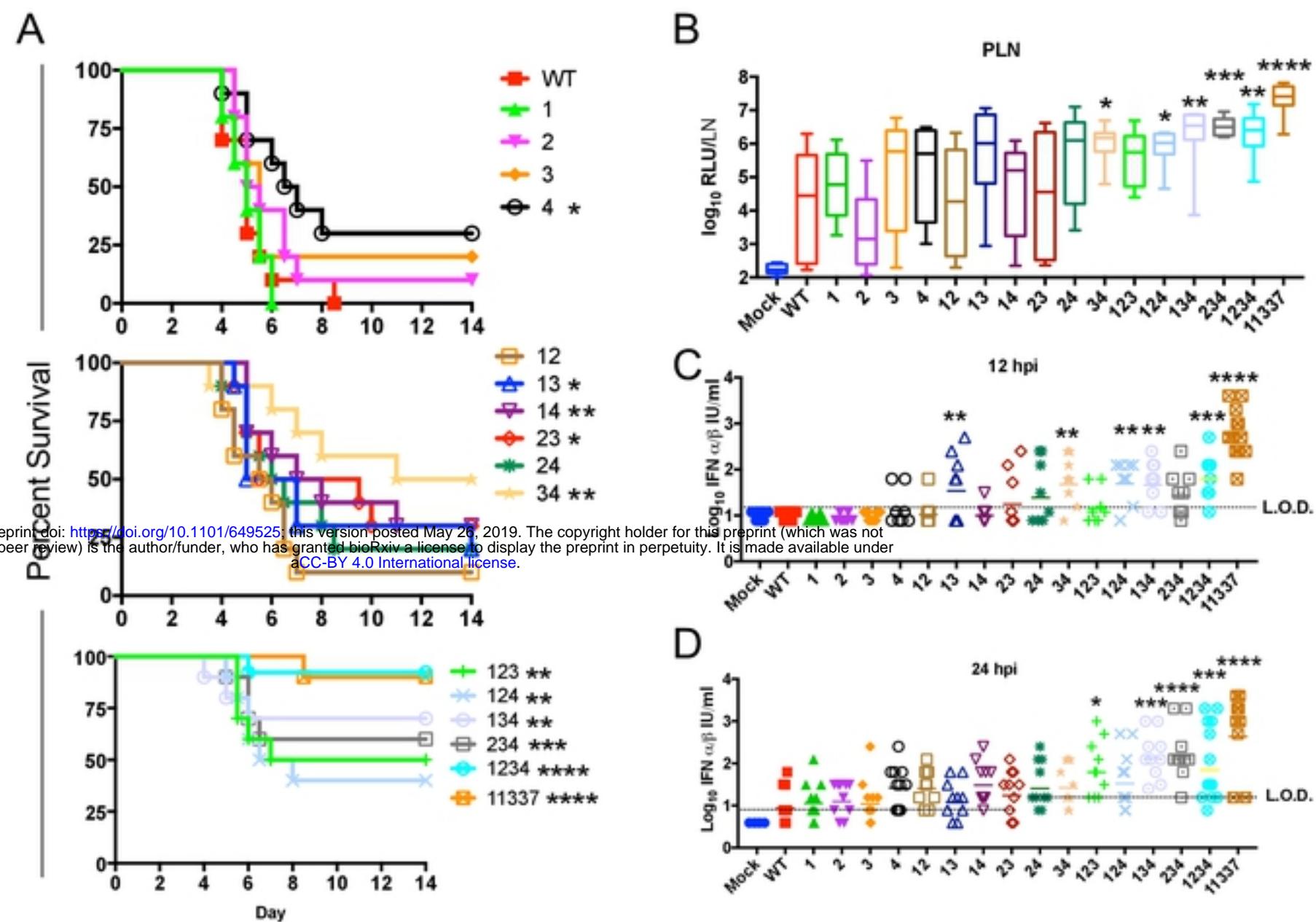
**Figure 1: miR-142-3p binds to EEEV 3' UTR in myeloid cells.** A) EEEV genome structure. miR-142-3p binds (red bar) to EEEV genome in the 3' UTR. Numbers indicate nucleotide position in the EEEV genome of the 3' UTR and miR-142-3p binding sites B) EEEV or EEEV 11337 translation reporters (7ug) were electroporated into the RAW monocyte/macrophage cell line. Anti-Ago1/2 antibody was used to immunoprecipitation of Argonaute proteins. qRT-PCR was used to quantify amount of reporter RNA in each immunoprecipitated fraction. Data is represented as fold change over mock. \*\*P<0.01, NS: not significant unpaired t test. n=2 independent experiments. Error bars represent SD. C) Biotin labeled miR-142-3p mimic or biotin labeled scramble (sc) were co-electroporated with the reporters into RAW cells. Streptavidin beads was used for immunoprecipitation and qRT-PCR was to quantify the amount of reporter RNA in each fraction. Data is represented as fold change over sc. n=2-3 independent experiments. Error bars represent SD. \*\*\*P<0.001, unpaired t test. D) Alignment of EEEV '3 UTR to mmu-miR-142-3p with red letter indicate mutated nucleotides (nt) in seed sequence. nt number indicates initial position in EEEV genome of miR-142-3p binding sites.

Figure 2



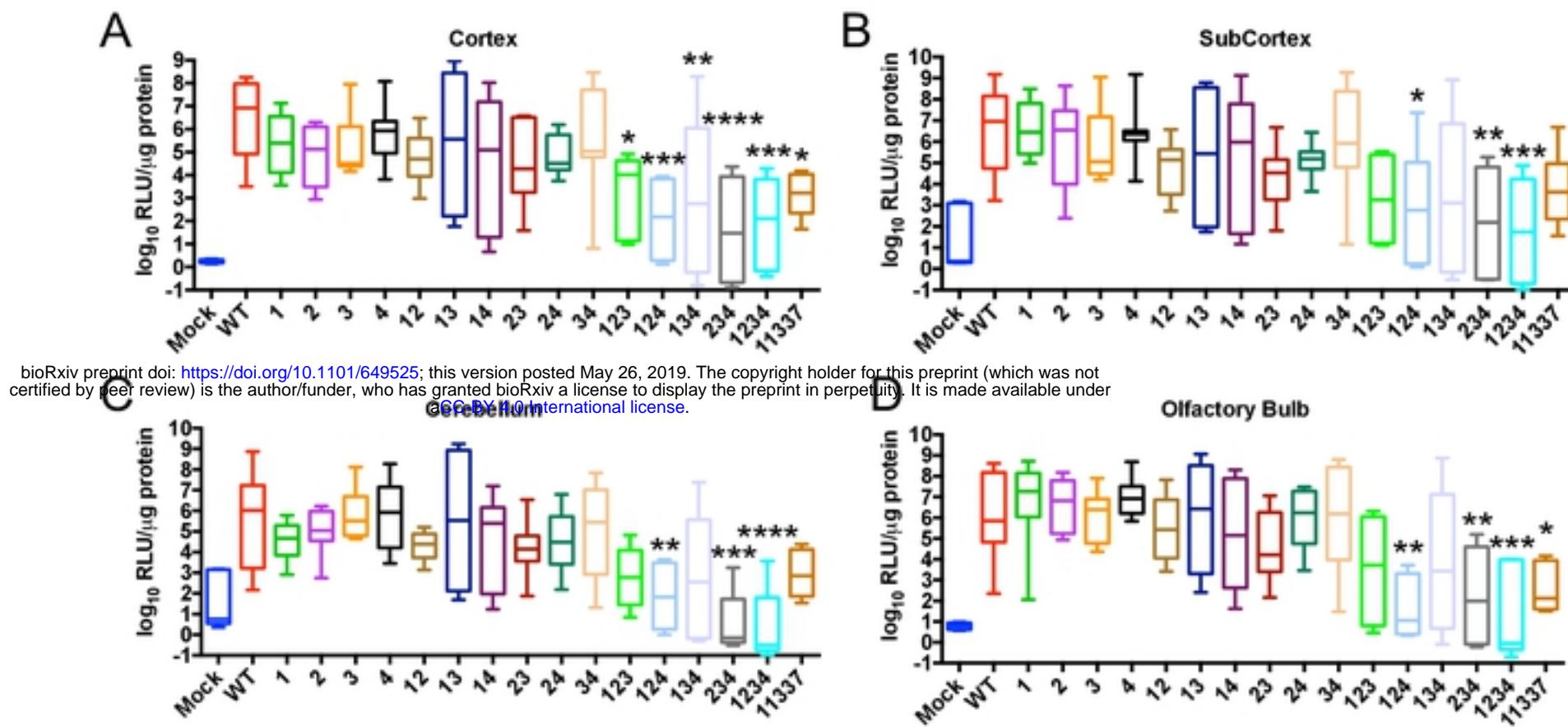
**Figure 2: Decreasing the number of miR-142-3p binding sites leads to increased virus replication in myeloid cells and viral attenuation *in vivo*.** A-C) Quantification of virus replication at 12 hpi. BHK (MOI=0.1), RAW (MOI=0.1), BMDCs (MOI=5) were infected with the EEEV mutants expressing nLuc. D-E) Virus replication in C6/36 mosquito cells (MOI=1) at (D) 8hpi and (E) 12hpi. Data is log<sub>10</sub> transformed and expressed as relative light units (RLU) per μg protein. N=6-9, 2-3 independent experiments. \*P<0.5, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 one way ANOVA with corrections for multiple comparisons using the Holm-Sidak method comparing each mutant to WT. Error bars represent SE.

Figure 3



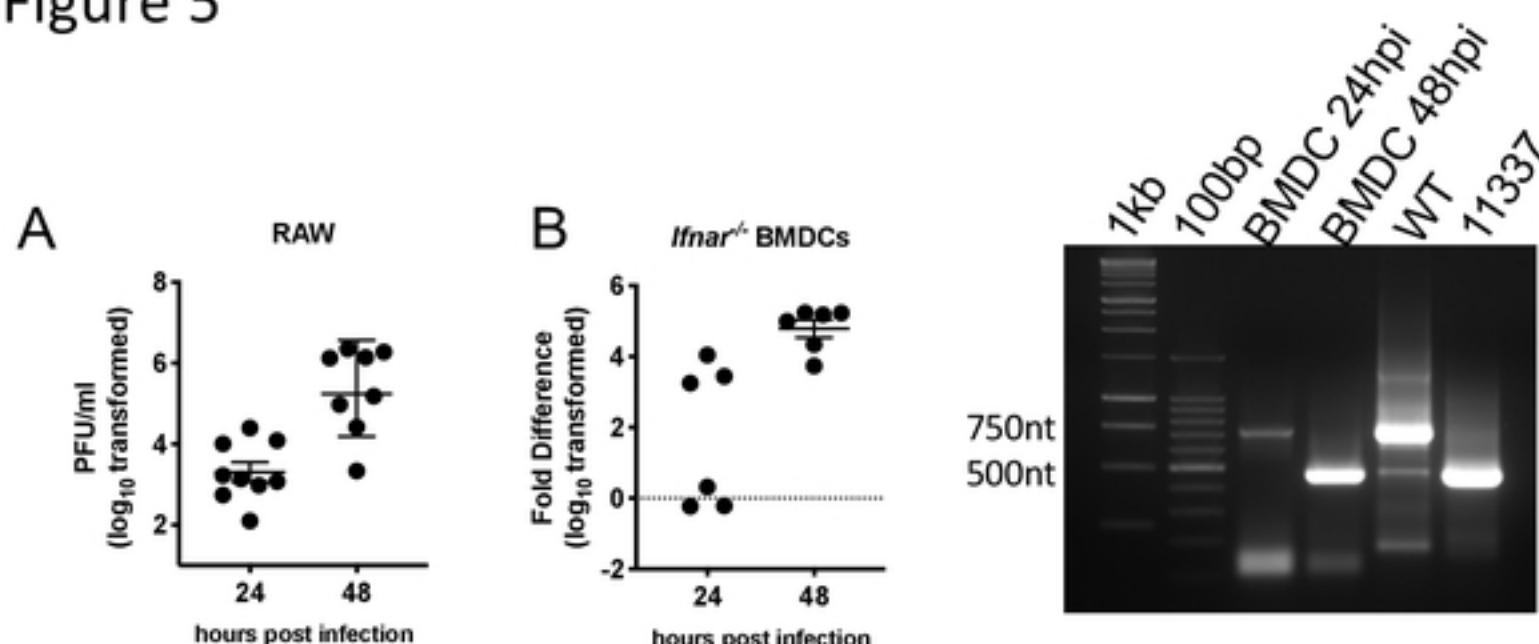
**Figure 3: Elimination of miR-142-3p binding sites leads to attenuation due to increased virus replication in popliteal lymph node and serum IFN.** A) Female outbred CD-1 mice (5-6 weeks) were infected with  $10^3$  pfu sc in each footpad. Morbidity and mortality were measured twice daily. n=10 mice from 2 independent experiments \*P<0.5, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001, Log-Rank Test comparing each EEEV mutant to WT. B) Outbred CD-1 mice were infected with  $10^3$  pfu of WT, 11337 or each mutant sc in each footpad. A single popliteal lymph node (PLN) was harvested at 12 hpi and analyzed for nLuc expression. Data is  $\log_{10}$  transformed and represented as RLU per LN. n = 7-12 mice, from 2-3 experiments. Box-and whisker plots represent min-max with bar representing the median value. C-D) Biologically active IFN- $\alpha/\beta$  levels in serum at 12 hpi (B) and 24 hpi (C). CD-1 mice were infected with  $10^3$  pfu of WT, 11337 or each mutant sc in each footpad. n=8-12 mice, from 2-3 experiments. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001, comparing the log-transformed data of each mutant with WT using the one way analysis of variance test with corrections for multiple comparisons using the Holm-Sidak method. LOD = limit of detection of the IFN assay. Bar represents geometric mean.

Figure 4



**Figure 4: Reduced virus replication in the central nervous system after infection with the triple and quadruple mutant EEEV viruses.** CD-1 mice were infected with  $10^3$  pfu of the EEEV mutants sc in each footpad. Tissues were harvested at 96 hours post infection. Virus replication in cortex (A), subcortex (B), cerebellum (C), and olfactory bulb (D). Data is  $\log_{10}$  transformed and represented as either RLU/LN or RLU per  $\mu\text{g}$  protein N=8 mice, from 2 independent experiments. \*P<0.5, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 one way analysis of variance test with corrections for multiple comparisons using the Holm-Sidak method comparing each mutant to WT. Box-and whisker plots represent min-max with bar representing the median value.

Figure 5

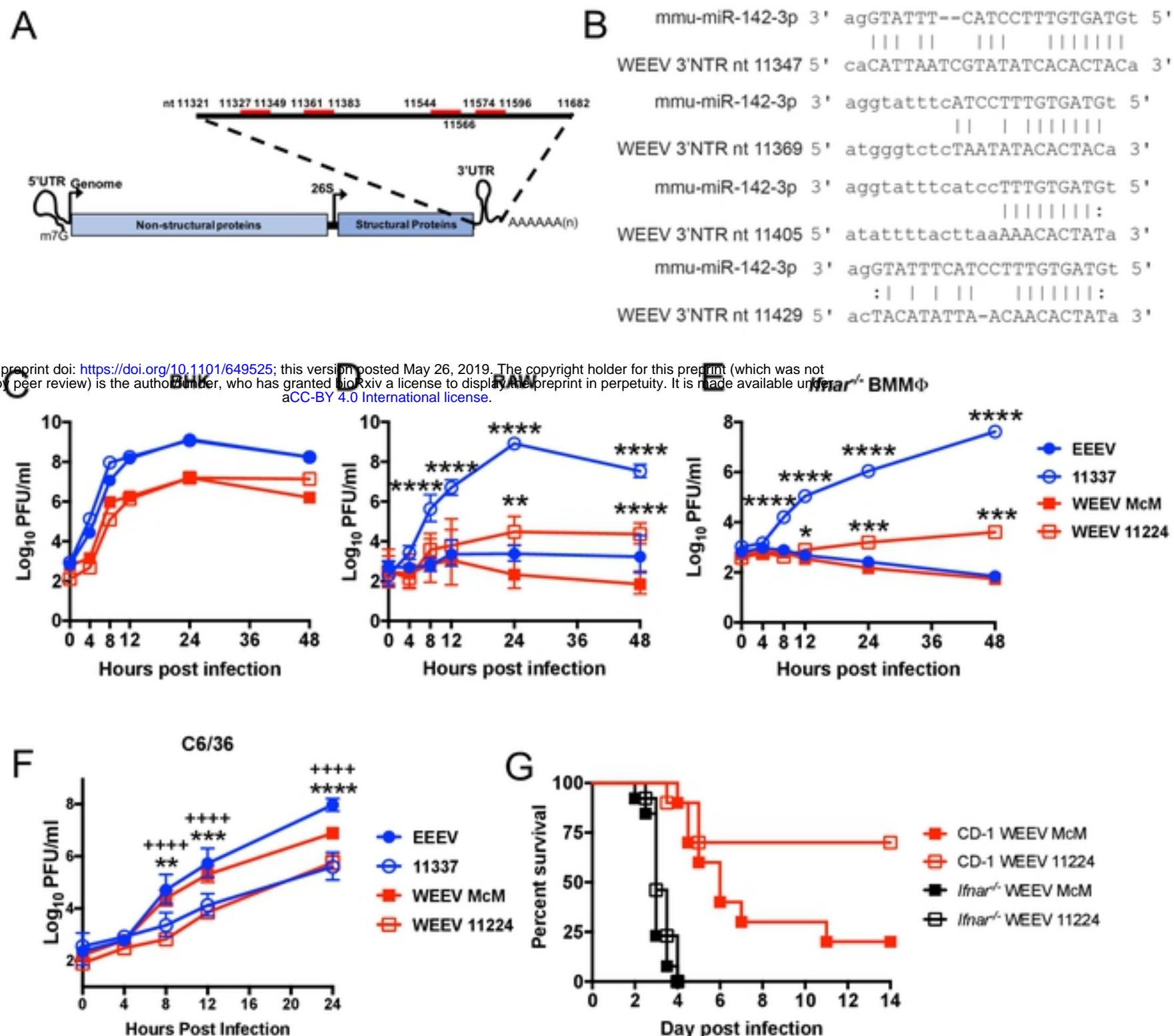


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**Figure 5: Escape of miR-142-3p suppression in myeloid cells. A-B)**

Replication of EEEV increases between 24 and 48 hpi *in vitro* detected by plaque assay in A) RAW cells or semi-quantitative RT-PCR in (B) *Ifnar*<sup>-/-</sup> BMDCs. n=6-9 individuals wells from 2-3 independent experiments. Fold difference in B is compared to mock samples. Bar represents geometric mean and geometric SD. C) Representative gel from PCR of EEEV 3' UTR of BMDC escape mutant between 24 hpi and 48 hpi. Control PCR product is approximately 750nt for WT EEEV and 500nt for 11337. 1kb and 100bp indicate 1kb and 100bp ladders respectively.

Figure 6



**Figure 6: WEEV McMillan (McM) encodes miR-142-3p binding sites that restrict myeloid cell replication.** A) Alignment in genome and location of miR-142-3p binding sites in WEEV McM 3' UTR. B) Alignments between murine mmu-miR-142-3p and WEEV 3'UTR. C-F) Viral growth curves in BHK (C), RAW (D), *Ifnar*<sup>-/-</sup> BMMΦ (E), or C6/36 (F) of wild-type viruses (filled) or miR-142-3p mutants (open). Multiplicities of infection: BHK and RAW MOI=1, *Ifnar*<sup>-/-</sup> BMMΦ MOI=5. n=6 from 2 independent experiments. Data was  $\log_{10}$  transformed and multiple unpaired t-tests were performed and corrected for multiple comparisons using the Holm-Sidak method. \*p<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001. +++++P<0.0001 comparing WEEV McM versus WEEV 11224 in C6-36 cells. Data is represented as geometric mean with geometric SD. G) Infection of outbred CD-1 mice (n=10 mice, 2 independent experiments) or *Ifnar*<sup>-/-</sup> mice (n=13 mice, 2 independent experiments) with  $10^4$  pfu of wild-type WEEV McM or mutant WEEV 11224 sc in both rear footpads. Morbidity and mortality were monitored twice daily. Mantel-Cox Log rank test was used to compare between viruses.