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1	The Capsid Protein of Semliki Forest Virus Antagonizes RNAi in
2	Mammalian Cells
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17 Running Title: SFV capsid antagonizes RNAi in mammalian cells

18 ABSTRACT

RNA interference (RNAi) is a conserved antiviral immune defence in eukaryotes, 19 20 and numerous viruses have been found to encode viral suppressors of RNAi (VSRs) to counteract antiviral RNAi. Alphaviruses are a large group of positive-stranded 21 22 RNA viruses that maintain their transmission and life cycles in both mosquitoes and mammals. However, there is little knowledge about how alphaviruses antagonize 23 RNAi in both host organisms. In this study, we identified that Semliki Forest virus 24 (SFV) capsid protein can efficiently suppress RNAi in both insect and mammalian 25 cells by sequestrating dsRNA and siRNA. More importantly, when the VSR activity 26 27 of SFV capsid was inactivated by reverse genetics, the resulting VSR-deficient SFV 28 mutant showed severe replication defects in mammalian cells, which could be rescued 29 by blocking the RNAi pathway. Besides, capsid protein of Sindbis virus (SINV) also inhibited RNAi in cells. Together, our findings show that SFV uses capsid protein as 30 VSR to antagonize RNAi in infected mammalian cells, and this mechanism is 31 probably used by other alphaviruses, which shed new light on the knowledge of SFV 32 and alphavirus. 33

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34 Importance

Alphaviruses are a genus of positive-stranded RNA viruses and include 35 36 numerous important human pathogens, such as Chikungunya virus, Ross River virus, Western equine encephalitis virus, etc, which create the emerging and re-emerging 37 38 public health threat worldwide. RNA interference (RNAi) is one of the most 39 important antiviral mechanisms in plants and insects. Accumulating evidence has provided strong support for the existence of antiviral RNAi in mammals. In response 40 to antiviral RNAi, viruses have evolved to encode viral suppressors of RNAi (VSRs) 41 to antagonize the RNAi pathway. It is unclear if alphaviruses encode VSRs that can 42 43 suppress antiviral RNAi during their infection in mammals. In this study, we first 44 uncovered that capsid protein encoded by Semliki Forest virus (SFV), a prototypic 45 alphavirus, had a potent VSR activity that can antagonize antiviral RNAi in the context of SFV infection in mammalian cells, and this mechanism is probably used by 46 other alphaviruses. 47

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RNAi is a conserved post-transcriptional gene silencing mechanism that 49 50 originally evolves as an intrinsic antiviral immune mechanism in a broad range of 51 eukaryotic organisms (1-3). In the process of antiviral RNAi, the viral replicative 52 intermediate double-stranded RNAs (vRI-dsRNAs) synthesized during virus replication are sensed and cleaved by host endoribonuclease Dicer into approximately 53 21- to 23-nucleotide (nt) virus-derived small interfering RNA (vsiRNA). These 54 vsiRNAs are then loaded into the Argonaute (AGO) protein of the RNA-induced 55 silencing complexes (RISCs) to mediate the cleavage of cognate viral genomic RNAs 56 (2, 4, 5). So far, RNAi has been widely recognized as the major antiviral response in 57 58 fungi, plants and insects (1, 2). For mammals, vRI-dsRNAs and other viral nucleic acids usually induce innate antiviral immunity such as the type I interferon (IFN-I) 59 system (1). Recently, accumulating evidence have provided strong support for the 60 existence of antiviral RNAi in mammals (6-10). 61

In response to antiviral RNAi, viruses have evolved to encode VSRs to antagonize 62 the RNAi pathway through different mechanisms (11). Some VSRs bind to and 63 sequestrate long dsRNAs and/or siRNAs to shield them from Dicer cleavage or to 64 65 prevent their loading into AGO, while others inhibit certain important components of the RNAi pathway such as Dicer or AGO (11). For instance, Flock house virus (FHV) 66 B2 acts as a VSR by preventing dsRNA from being cleaved by Dicer-2 as well as 67 sequestering the siRNAs produced by Dicer-2 (12-14), while Wuhan nodavirus B2 68 69 can directly bind and inhibit Drosophila Dicer-2 required for vsiRNA production (15,

16). Besides, cricket paralysis virus 1A directly inhibits the endonuclease activity of
AGO2 and simultaneously targets AGO2 for proteasomal degradation in *Drosophila*(17).

73 In mammals, a number of viral proteins, such as Ebola virus VP35 (18), HIV-1 74 Tat (19), Hepatitis C virus core (20), Dengue virus NS4B (21), Yellow Fever virus (YFV) capsid (22), and coronavirus 7a and nucleocapsid (23, 24), have been shown to 75 suppress ectopic dsRNA/shRNA-induced RNAi in vitro. On the other hand, a handful 76 of viral proteins encoded by mammalian viruses, including Enterovirus A71 (EV-A71) 77 3A, Influenza A virus NS1, and Nodamura virus (NoV) B2, have been found to act as 78 79 bona fide VSRs that antagonize antiviral RNAi in the course of viral infections (6, 8, 80 10).

Alphaviruses are a large group of positive-stranded RNA viruses that belong to 81 the genus Alphavirus in the family Togaviridae (25), and include numerous medically 82 important human pathogens such as Sindbis virus (SINV), Chikungunya virus 83 (CHIKV), Ross River virus, Eastern equine encephalitis virus, Western equine 84 encephalitis virus, Venezuelan equine encephalitis virus, etc. The infections by these 85 viruses are responsible for a broad spectrum of diseases, ranging from mild, 86 87 undifferentiated, febrile illness to debilitating polyarthralgia, encephalitis, and even death in human and horses (26-29). To date, there is no approved antiviral therapy 88 specific for alphaviruses (30). Alphaviruses transmit between mosquito vectors and 89 vertebrate hosts (31, 32), and create emerging and re-emerging public health threat 90 worldwide (33). Although previous studies indicated the critical role of antiviral 91

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94 antiviral RNAi during viral infection in mammals. SFV is a member of the Alphavirus genus. Although SFV infection only causes a 95 96 mild febrile illness in human, it is highly pathogenic in rodents and serves a model virus to investigate the mechanisms of viral replication, virus-host interaction, and 97 innate immunity (34-36). SFV contains a single positive-stranded RNA genome of 98 approximately 12 kb, which consists of two open reading frames (ORFs) that encode 99 four non-structural proteins (nsP1 to nsP4), three structural proteins (capsid, envelope 100 101 glycoproteins E1 and E2), and two small cleavage products (E3 and 6K) (36). Both 102 ORFs are translated as polyproteins, which undergo *cis* and *trans* cleavage to form the 103 mature viral proteins. SFV capsid protein is multifunctional and plays a critical role in the encapsidation of genome and formation of viral nucleocapsid capsid (37-39). In 104 this study, we first uncovered that SFV-encoded capsid protein had a potent in vitro 105 VSR activity that suppressed artificially induced RNAi in both insect and mammalian 106 107 cells. We further demonstrated that SFV capsid can act as bona fide VSR to antagonize RNAi in the context of SFV infection in mammalian cells. 108

RNAi in regulating the replication of alphaviruses such as CHIKV and SINV in

mosquitoes (31), it is unclear if alphavirus encodes a *bona fide* VSR that can suppress

109 **RESULTS**

110 SFV capsid protein is a potential VSR.

111 To evaluate whether SFV encodes any protein that works as a potential VSR, we 112 examined all SFV-encoded proteins via a reversal-of-silencing assay in Drosophila S2 113 cells, which was previously used by us to screen VSRs of other viruses (15). In brief, cultured S2 cells were co-transfected with the plasmid encoding EGFP and 114 EGFP-specific dsRNA, which is cleaved by fly Dicer-2 to produce siRNA and induce 115 RNAi, together with the plasmid encoding one of the SFV proteins (Fig. 1A). The 116 expression of the viral proteins was confirmed by Western blotting with anti-His 117 118 antibody (Fig. 1B). At 48 hour post transfection (h.p.t.), the mRNA levels of EGFP 119 were detected by Northern blotting with a DIG-labeled RNA probe targeting 520-700 120 nt of EGFP ORF. The EGFP-specific dsRNA can induce RNAi to destruct EGFP 121 transcript (Fig. 1A, lane 2). FHV B2 (FB2), a well-characterized VSR, was used as a positive control, which expectedly restored EGFP mRNA levels (Fig. 1A, lane 3). Our 122 data show that the ectopic expression of SFV capsid protein effectively restored the 123 accumulation of EGFP mRNA (Fig. 1A, lane 8), indicating that SFV capsid protein is 124 a potential VSR. 125

To further confirm the VSR activity of SFV capsid, we examined whether capsid could rescue the replication of a B2-deficient FHV RNA1 replicon (FR1 Δ B2) in S2 cells (13). This mutant replicon lost the ability to suppress RNAi, leading to a defective self-replication of FHV RNA1 and subgenomic RNA3 in S2 cells (Fig. 1C, compared lanes 1 and 2). Our data show that the replication defect could be partially

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132	and 4) or by the knockdown of fly AGO2 or Dicer-2 (Fig. 1C, lanes 5 and 6).
133	Because that RNAi pathway is conserved from insects to mammals, we sought to
134	determine whether SFV capsid can suppress RNAi in cultured human 293T cells via
135	the reversal-of-silencing assay, in which RNAi is induced by co-expressing EGFP
136	expression vector and EGFP-specific short hairpin RNA (shRNA). Our data show that
137	ectopic expression of SFV capsid effectively suppressed the shRNA-induced RNAi in
138	293T cells (Fig. 1D and E). Moreover, it was expectedly that ectopically expressing
139	NoV B2 (NB2), another well-established VSR, suppressed RNAi (Fig. 1D, lane 3).
140	Together, our data demonstrate that SFV capsid protein is a potential VSR that can
141	suppress RNAi in both insect and mammalian cells.
142	Capsid proteins from alphaviruses share high homology in amino acid sequences,
143	suggesting a conserved function (40). Thus, we examined the VSR activity of capsid
144	protein from SINV via the reversal-of-silencing assays in both Drosophila S2 and

rescued by the ectopic expression of either FHV B2 or SFV capsid (Fig. 1C, lanes 3

both cells (Fig. 1F and G), suggesting that the in vitro VSR activity is a common 146 147 feature for alphaviral capsid proteins.

human 293T cells, respectively. Our data show that SINV capsid inhibited RNAi in

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SFV capsid suppressed Dicer-mediated siRNA production by sequestrating 149 dsRNA. 150

It is well established that dsRNA/shRNA-induced RNAi requires the 151 Dicer-mediated cleavage of dsRNA or shRNA into siRNA (1). To explore whether 152

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158	compared to that in cells overexpressing empty vector, indicating that SFV capsid can
159	inhibit Dicer-mediated siRNA production. These results are consistent with the
160	observation that SFV capsid could effectively restore shRNA-mediated elimination of
161	EGFP transcript in 293T cells (Fig. 1D).
162	We sought to examine whether SFV capsid inhibits siRNA production by directly
163	binding to long dsRNA. To this end, we purified the recombinant MBP-fusion capsid
164	protein (MBP-capsid, Fig. 2B, lane 2) and conducted EMSA by incubating the in vitro
165	transcribed DIG-labeled 200-nt dsRNA together with MBP-capsid. As shown in Fig.
166	2C, capsid protein can bind to dsRNA directly and the shifting amount of labeled
167	dsRNAs was increased with the increasing amounts of MBP-capsid used in the
168	reaction. MBP-fusion FHV B2 (MBP-FB2) was used as positive control.
169	Subsequently, we sought to examine whether SFV capsid can protect dsRNA from
170	Dicer cleavage by using an in vitro RNase III assay (41). RNase III was widely used
171	as the Dicer substitute to examine the activities of VSRs as previously described (16).
172	Our data show that the presence of MBP-capsid efficiently protected dsRNA from
173	RNase III digestion in a dose-dependent manner (Fig. 2D, lanes 5-8), while dsRNA

SFV capsid can suppress this process, RNAs harvested from 293T cells co-expressing

EGFP expression vector and EGFP-shRNA together in the presence or absence of

SFV capsid were subjected to small RNA Northern blotting by using a DIG-labeled

RNA oligo probe targeting EGFP siRNA. As shown in Fig. 2A, the accumulation of

22-nt Dicer-cleaved siRNA product was reduced in cells overexpressing SFV capsid

174 was cleaved into siRNA in the presence of MBP alone (Fig. 2D, lane 2).

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SFV capsid suppressed siRNA-induced RNAi. 178

179 In the RNAi pathway, when being processed from dsRNA, siRNAs are incorporated into RISC to mediate the cleavage of cognate mRNAs (1). Since we 180 have found that SFV capsid can inhibit RNAi by sequestrating dsRNA, it would be 181 intriguing to examine whether SFV capsid could also suppress siRNA-induced RNAi, 182 which is the step of post siRNA biogenesis. To this end, 293T cells were 183 184 co-transfected with a plasmid expressing EGFP and chemically synthesized 185 EGFP-specific siRNA (siEGFP), together with the expression vector for SFV capsid. 186 As shown in Fig. 3A, the chemically synthesized siRNA mediated the silencing of EGFP mRNA, while SFV capsid efficiently restored the level of EGFP mRNA, 187 indicating that SFV capsid can suppress siRNA-induced RNAi in cells. 188

Because SFV capsid can suppress siRNA-induced RNAi, we speculate that 189 capsid protein may have siRNA-binding activity. To test this possibility, we 190 conducted EMSA by incubating purified MBP-capsid together with DIG-labeled 191 192 synthetic 22-nt siRNA. Our data show that SFV capsid protein can directly bind to siRNA in a dose-dependent manner (Fig. 3B). 193

Taken together, our findings indicate that SFV capsid can suppress RNAi by 194 sequestrating both dsRNA and siRNA. 195

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197 K124/K128 and K139/K142 of SFV capsid are critical for the VSR activity.

After determining the in vitro VSR activity of SFV capsid, we sought to identify 198 199 the critical domain or amino acid (aa) required for its VSR activity. Previous studies 200 showed that SFV capsid consists of an N-terminal segment (aa 1-118) and a 201 C-terminal protease domain (aa 119-267) formed by two β -sheet domains (aa 119-181 202 and aa 182-267) (40). Accordingly, we constructed a set of capsid protein truncations, as illustrated in Fig. 4A, and examined their activities to suppress RNAi via the 203 reversal-of-silencing assay in S2 cells. Our data show that the region of aa 119-181 is 204 critical for capsid's VSR activity (Fig. 4A). The expression of capsid protein 205 206 truncations was confirmed by Western blotting with anti-His antibody (Fig. 4B).

207 To identify the critical residue(s) within the aa 119-181 region required for the VSR activity, we performed multiple sequences alignments of capsids encoded by 208 RRV, SFV, SINV and CHIKV. The conserved positively charged residues, lysine (K) 209 210 and aspartic acid (D), were subjected to single-point or double-point mutations to Alanine (A), and the resulting mutant capsid proteins were then examined via the 211 reversal-of-silencing assay in S2 cells. Although none of the single-point mutations 212 disrupted the VSR activity (Fig. 4C and D), the K124A/K128A or K139A/K142A 213 214 mutation (capsid_{K124A/K128A} or capsid_{K139A/K142A}) significantly suppressed the activity of SFV capsid to suppress RNAi (Fig. 4E, lanes 7 and 8; Fig. 4F and G) in S2 cells. In 215 216 addition, the truncation mutations (M2 and M3) and capsid_{K124A/K128A} or capsid_{K139A/K142A} also lost the VSR activity in 293T cells (Fig. 4H and I). We also 217 218 found that the K124A/K128A or K139A/K142A mutation significantly 219

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(Fig. 4J and K).

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222 abolished the dsRNA- and siRNA-binding activities of SFV capsid (Fig. 5A and B). 223 Interestingly, although capsid_{K124A/K128A} and capsid_{K139A/K142A} failed to bind to dsRNA or siRNA, their activities to bind to ssRNA were still intact (Fig. 5C). 224 Together, these data show that the K124A/K128A and K139A/K142A residues 225 are critical for the dsRNA/siRNA-binding and VSR activities of SFV capsid. 226 227 Construction and recovery of VSR-deficient SFV. 228 229 To find out whether SFV capsid protein indeed suppresses antiviral RNAi during virus infection, we introduced the K124A/K128A and K139A/K142A mutation into 230 the capsid coding region of the infectious clone of SFV (Fig. 6A). The wild-type 231 (SFV_{WT}) and K124A/K128A mutant (SFV_{K124A/K128A}) viruses were successfully 232 recovered and the plaque morphology of these two viruses was similar (Fig. 6B), 233 whereas K139A/K142A mutant virus displayed lethal phenotype. 234 Because alphaviral capsid protein is critical for the formation of nucleocapsid 235 236 and virion maturation, we sought to exclude the possibility that the K124A/K128A mutation may affect other important functions of SFV capsid, such as the process of 237 238 virion assembly and the virus entry into cells. Our previous data showed that SFV capsid_{K124A/K128A} still kept the ssRNA-binding activity (Fig. 5C), implying that the 239

suppressed the activity of SFV capsid to suppress siRNA-induced RNAi in 293T cells

Moreover, we found that either K124A/K128A or K139A/K142A mutation

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important for nucleocapsid assembly, is not affected. Moreover, we purified the
virions of SFV_{WT} and SFV_{K124A/K128A}, and then examined the morphology of both WT
and mutant virions via transmission electron microscopy (TEM). Our results showed
that the K124A/K128A mutation did not affect the morphology and diameter of viral
particles (Fig. 6C).

Subsequently, we sought to determine whether the K124A/K128A mutation 246 affected the entry efficiency of SFV into cells. To this end, 293T cells were infected 247 with SFV_{WT} or SFV_{K124A/K128A} at a multiplicity of infection (MOI) of 5, and at 15, 30 248 and 45 min post infection, the viruses remaining in the supernatant and the viral 249 250 RNAs within cells were examined by plaque assays and qRT-PCR, respectively. Our 251 data show that the levels of the remaining virions in the supernatant and the viral 252 RNAs within cells of $SFV_{K124A/K128A}$ were comparable to those of SFV_{WT} at each time 253 point (Fig. 6D), indicating that the K124A/K128A mutation did not affect the entry of 254 SFV into cells.

After determining that the K124A/K128A mutation did not affect the virion assembly and viral entry of SFV, we determined the one-step growth curve of WT and mutant viruses in human 293T cells, showing that $SFV_{K124A/K128A}$ exhibited weaker growth patterns than did SFV_{WT} in 293T cells (Fig. 6E). This result indicates that the inactivation of VSR function led to the restricted replication of SFV.

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The replication defect of VSR-deficient SFV can be rescued by the deficiency of
RNAi in 293T cells.

263	To further explore the VSR function of SFV capsid during viral infection, 293T
264	cells were infected with WT or VSR-deficient SFV, and viral RNA accumulation
265	were determined at 6, 12, 24 hour post infection (h.p.i.), respectively. As expected, the
266	viral RNA accumulation of $SFV_{\rm K124A/K128A}$ was lower than that of $SFV_{\rm WT}$ in 293T
267	cells (Fig. 7A). The genetic ablation of the RNAi pathway by Dicer knockout in 293T
268	cells (NoDice) rescued the replication of $SFV_{K124A/K128A}$ (Fig. 7A). Interestingly, the
269	RNA accumulation of $SFV_{K124A/K128A}$ was significantly reduced in 293T cells treated
270	with enoxacin compared to that in the controlled 293T cells (Fig. 7B). Of note,
271	enoxacin is a well-known RNAi enhancer and functions at steps post siRNA
272	production by Dicer in the RNAi pathway (9, 42). Expectedly, ectopic expression of
273	human Dicer (hDicer) in 293T-NoDice cells resulted in the reduced RNA
274	accumulation of $SFV_{K124A/K128A}$ (Fig. 7B and C).

Moreover, the viral RNA replication of $SFV_{K124A/K128A}$ in 293T cells was increased by the ectopic expression of SFV capsid or NoV B2, but not the VSR-deficient mutant of capsid (capsid_{K124A/K128A}) or NoV B2 (B2_{R59Q}, named as mB2) (Fig. 7A and D). And the rescuing effect of ectopically expressed capsid on the replication of VSR-deficient SFV was confirmed by using Northern blotting with a DIG-labeled RNA probe that recognized both genomic and subgenomic RNAs of SFV (Fig. 7E). Downloaded from http://jvi.asm.org/ on November 8, 2019 at Univ of Nottingham

Furthermore, we found that ectopic expression of NoV B2 or SFV capsid could not rescue the RNA accumulation of $SFV_{K124A/K128A}$ in 293T-NoDice cells (Fig. 7F and G). These results indicate that the rescuing effect of ectopically expressed foreign

286	To exclude the potential impact of IFN-I response, we treated 293T or
287	293T-NoDice cells infected by $SFV_{K124A/K128A}$ with Ruxolitinib, a JAK1 and JAK3
288	inhibitor, to block IFN-I. We found that ectopic expression of foreign VSRs or
289	deficiency of Dicer could still enhance the RNA accumulation of $SFV_{\rm K124A/K128A}$ in
290	Ruxolitinib-treated cells (Fig. 7H and I), confirming that the rescuing effect is
291	irrespective of IFN-I.

In addition, we examined the production of vsiRNAs in SFV_{K124A/K128A}-infected 292 Aedes mosquito Aag2 cells. The Deficiency of VSR also resulted in substantial 293 294 attenuation of viral RNA accumulation of SFV_{K124A/K128A} in Aag2 cells (Fig. 7J), 295 consistent with the findings in 293T cells. SFV vsiRNAs in Aag2 cells were detected 296 via Northern blotting with RNA probe complementary to 3'-end 1-50 nt of the 297 negative-stranded antigenomic RNA. Our findings showed that the production of SFV vsiRNAs was dramatically enhanced in Aag2 cells infected with SFVK124A/K128A but 298 not SFV_{WT} (Fig. 7K). 299

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301 DISCUSSION

Alphaviruses infect and replicate in both invertebrate vectors and mammalian hosts. Efficient transmission of these viruses depends on their activities to counteract the antiviral immune response in both mosquito vectors and human hosts (31). RNAi is an antiviral immune response conserved in both invertebrates and mammals, while many viruses encode VSRs as the countermeasure (2, 5). However, it was still unclear if alphavirus encodes a VSR to antagonize antiviral RNAi in the context of viral
infection in mammalian cells. To date, the identification of a *bona fide* VSR encoded
by a mammalian virus requires to answer the following questions: 1) if disabling VSR
by reverse genetic can cause viral replication defect during authentic viral infection; 2)
if the genetic ablation of the RNAi pathway can rescue the replication defect of the
VSR-disabled mutant virus.

In the present study, we screened the viral proteins of SFV, a prototypic 313 alphavirus, for VSR activity in insect and mammalian cells. We found that SFV 314 capsid protein possesses VSR activity. The in vitro EMSA analysis shows that SFV 315 capsid has both dsRNA- and siRNA-binding activities that are indispensable for its 316 317 VSR function. More importantly, the VSR deficiency of SFV capsid resulted in substantial restriction of viral replication in mammalian cells. And this 318 defective-replication of VSR-deficient mutant SFV can be rescued by the deficiency 319 of RNAi by either ectopically expressing foreign VSRs or blocking the RNAi 320 pathway, which is irrespective of the IFN-I system. In addition, when the 321 322 capsid-mediated RNAi suppression was genetically disabled in SFV, the production of vsiRNAs was enhanced in infected mosquito cells. Thus, we propose a hypothesis 323 324 that SFV capsid may protect viral RNA from antiviral RNAi at two stages: (i) 325 blocking vRI-dsRNA from Dicer cleavage through dsRNA-binding activity; and (ii) binding to vsiRNA to suppress in the incorporation of vsiRNA to RISC (Fig. 8). 326

327 In addition to SFV capsid, a remaining question is that if alphaviruses encode 328 another VSR(s). Previous study has identified CHIKV nsP2 and nsP3 suppressed lournal of Virology

shRNA-induced RNAi in insect and mammalian cells (43). However, the potential 329 VSR activity of nsP2 and nsP3 was not tested in the context of an authentic viral 330 331 infection. Over-expression of any protein with an effective dsRNA- or siRNA-binding 332 activity may suppress RNAi in some cases, which does not mean that it acts as a VSR 333 during viral infection. In addition, viruses may encode more than one VSR, as seems to be the case for some plant viruses, such as citrus tristeza virus (p20, p23 and coat 334 protein) (44) and potyviruses (P1 and HcPro) (45). 335

Alphaviral RNA packaging requires active viral RNA synthesis, implying that the 336 replication and encapsidation are tightly connected (46). This may explain why only 337 the genomic RNA can be specifically packaged into the virion, and suggests that 338 339 alphaviral structural proteins could directly involve in viral RNA replication. Indeed, 340 SFV capsid is a versatile structure protein that can bind to the large ribosomal subunit 341 in the cytoplasm and associate with the viral RNAs to form the viral nucleocapsid (38, 342 39). In this current study, we provided evidence that SFV capsid is a potent VSR, which plays an important role in the viral RNA replication in insect and mammalian 343 cells. Our findings are consistent with the previous findings that VSR activities were 344 observed in viral structural coat proteins encoded by numerous viruses, including 345 346 Turnip crinkle virus coat protein, YFV capsid protein and coronavirus nucleocapsid protein, and their VSR activities have been found to be associated with their 347 dsRNA-binding capacities (22, 24, 47). 348

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SFV capsid is made up of two domains, the RNA binding N-terminal segment 349 350 (aa 1-118) and the C-terminal globular protease domain (aa 119-267), which is critical

for the dimerization of capsid (40). Indeed, our mutational analyses showed that 351 mutations of K124A/K128A or K139A/K142A abolished the dsRNA-/siRNA-binding 352 353 of SFV capsid but not ssRNA-binding activities. This data indicates that these 354 residues are not directly involved in the association of SFV capsid with viral genomic 355 RNAs, but possibly in the process of protein dimerization. Our results are consistent with the previous findings that dimerization is required for RNAi suppression 356 activities of many VSRs, such as NoV B2 and EV-A71 3A (8, 48). The future work 357 will demonstrate the relationship between the VSR activity and the dimerization of 358 SFV capsid. 359

360 In summary, our study demonstrates that SFV capsid functions as a VSR in 361 facilitating viral replication by blocking either dsRNA from Dicer cleavage or 362 vsiRNA loading into RISC in mammalian cells. Moreover, it shows for the first time 363 that an alphavirus can antagonize antiviral RNAi in the context of viral infection, 364 extending our knowledge about the interaction between alphavirus and antiviral RNAi 365 immunity.

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368 MATERIALS AND METHODS

Plasmids and RNAs 369

For the expression of SFV proteins, their ORFs were cloned into insect expression 370 vector pAc5.1/V5-HisB, respectively. To express proteins in 293T cells, the ORF of 371 SFV capsid was constructed into the pRK-Flag. The full-length cDNA of the FHV 372

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RNA1 and FHV RNA1 ΔB2 were described previously (24). For the purification of 373 the MBP fusion capsid protein, its ORF was inserted into the pMAL-c2X vector. The 374 375 enhanced green fluorescent protein (EGFP)-siRNA (siEGFP) was chemically synthesized by Rui Bo, Guangzhou, China. The full-length SFV cDNA clone (strain 376 377 SFV4) was constructed in pCMV-Myc vector driven by CMV promoter (kindly provided by Dr. Tero Ahola, University of Helsinki). 378

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Cell culture 380

HEK293T cells were maintained in Dulbecco modified Eagle medium (DMEM) 381 382 supplemented with 10% fetal bovine serum (FBS) (GIBCO), 100 U/ml penicillin and 383 100 µg/ml streptomycin at 37°C in an incubator with 5% CO₂. The 293T-NoDice cell line was kindly provided by Dr. Bryan R. Cullen (Durham, NC, USA). Drosophila S2 384 or Aag2 cells were cultured in Schneider insect medium with 10% FBS at 27°C. 385 Before transfection with FuGene HD reagent (Roche, Basel, Switzerland), the 386 medium was changed to DMEM or Schneider insect medium containing 2% FBS 387 without any antibiotic. Cells were harvested at 48 h.p.t.. 388

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390 Construction and recovery of SFV mutant virus

To construct the SFV mutant clone, we obtained 6636-8748 nt of the full-length 391 cDNA clone via PCR, and cloned it into the pMd-18T vector. Then, the 392 K124A/K128A and K139A/K142A mutations were introduced into this plasmid via 393 over-lap PCR. The resulting mutant fragments were inserted into the full-length 394

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cDNA clone by double enzyme digestion (Sbf I and Xbal I). These infectious cDNA 395 clones were then transfected into the 293T cells using the FuGene HD reagent (Roche, 396 397 Basel, Switzerland), and the rescued viruses were harvested 48 h.p.t.. The virus titers 398 were measured by plaque assays.

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Western blotting 400

Cells were harvested in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% 401 402 NP-40, 0.25% deoxycholate and a protease inhibitor cocktail (Rhochel)]. Then the lysates were subjected to 12% SDS-PAGE and Western blotting according to our 403 404 standard procedures (49). The antibodies used in this study are as follow: anti-Tubulin 405 (Protein Tech Group, 1:3000), anti-His (Protein Tech Group, 1:10000), anti-Flag 406 (Protein Tech Group, 1:5000), anti-Dicer (Protein Tech Group, 1:2000).

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Northern blotting and qRT-PCR 408

Total RNAs were extracted using Trizol reagent (Thermo) according to the 409 manufacturer's instructions. For the detection of EGFP mRNA, 5 µg of total RNAs 410 were subjected to denatured 1.5% agarose gels with 2.2 M formaldehyde. The 411 412 separated RNAs were transferred onto the Hybond-A nylon membrane (GE Healthcare) and fixed by 120°C for 15 min. Then the membranes were hybridized 413 with digoxigenin (DIG)-labeled probes in Hybridization Ovens at 65°C overnight. 414 The membranes were then incubated with anti-DIG antibody conjugated with alkaline 415 phosphatase, and exposed to the luminescent image analyzer LAS4000 (Fuji Film). 416

Sos		
ipt F	417	The probes for detection of EGFP, Rp49 and GAPDH mRNA were complementary to
uscr	418	520-700 nt, 273-490 nt and 760-1060 nt of their ORF regions, respectively. The probe
Aan	419	for detection FHV RNA1 and subgenomic RNA3 were complementary to 2738-3058
ed >	420	nt of B2 coding region. The probe for detection SFV genome and subgenomic RNA
Accepted Manuscript Pos	421	were complementary to 723-1314 nt of E2 protein coding region. These probes were
Ace	422	labeled with DIG-UTP (Roche) by in vitro transcription. For detection of small RNAs,
	423	20 μ g of total RNAs were subjected to 7 M urea-15% PAGE and transferred to
	424	Hybond-A nylon membrane (GE Healthcare). The membrane was chemically
	425	cross-linked in 1-ethly-3-(3-dimethylaminopropyl) carbodiimide (EDC) at 60°C. For
	426	detection of vsiRNA, Aag2 cells were infected with SFV_{WT} or $SFV_{K124A/K128A}$ at an
, do	427	MOI of 10 for 24 h.p.t., then the total RNAs were subjected to 7 M urea-15% PAGE
of Virology	428	for Northern blotting analysis. The probes targeting EGFP siRNA, U6 and vsiRNA

FHV RNA1 and subgenomic RNA3 were complementary to 2738-3058 ing region. The probe for detection SFV genome and subgenomic RNA mentary to 723-1314 nt of E2 protein coding region. These probes were DIG-UTP (Roche) by in vitro transcription. For detection of small RNAs, tal RNAs were subjected to 7 M urea-15% PAGE and transferred to ylon membrane (GE Healthcare). The membrane was chemically in 1-ethly-3-(3-dimethylaminopropyl) carbodiimide (EDC) at 60°C. For vsiRNA, Aag2 cells were infected with SFV_{WT}or SFV_{K124A/K128A} at an or 24 h.p.t., then the total RNAs were subjected to 7 M urea-15% PAGE blotting analysis. The probes targeting EGFP siRNA, U6 and vsiRNA were synthesized by Takara, and their sequences were listed in Table 1. qRT-PCR 429 using SYBR mix (Takara) was carried out to detect the expression of EGFP mRNA, 430 β-actin mRNA and SFV NS1 mRNA. The primes and oligonucleotides used in this 431 study are shown in Table 1. 432

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434 Expression and purification of capsid protein

The coding regions of SFV capsid protein and FHV B2 were cloned into pMAL-c2X. 435 Then Escherichia coli BL21 (Invitrogen) transformed with the expression plasmids 436 were grown to the log phase at 37°C and induced with 0.8 mM IPTG 437 (isopropyl- β -D-thiogalactopyranoside) at 22°C for 6 h. Thus, cells were harvested by 438

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centrifugation and resuspended in lysis buffer [1 M Tris-HCl (pH 7.5), 0.2 M NaCl, 439 0.5 M EDTA (pH 8.0), 10 mM β -mercaptoethanol, 5% absolute ethylalcohol, 10% 440 441 glycerinum] and followed by sonication and centrifugation to remove the debris. 442 Finally, the proteins in the supernatant were purified using Amylose Resin (New 443 England BioLabs) according to the manufacturer's instructions.

444

Electrophoretic mobility shift assay (EMSA) and RNase III cleavage assays 445

MBP-fusion capsid was reacted with DIG-labeled RNAs (0.5 µM 200-nt dsRNA, 0.5 446 µM 200-nt ssRNA or 1 µM 22-nt siRNA.) in a reaction buffer containing 40 mM 447 MgCl₂, 50 mM NaCl, 25 mM HEPES (pH 7.5), 3 mM dithiothreitol (DTT) and 1 U of 448 449 RNase inhibitor; the total volume is 10 μ L. After incubation for 30 min at 25°C, the reaction mixtures were subjected to 1.5% native-TBE agarose gel and then transferred 450 to Hybond-A nylon membrane (GE Healthcare). The membranes were washed with 451 452 maleic acid buffer for 10 min, and then incubated with anti-DIG antibody conjugated with alkaline phosphatase (Roche) for 30 min. 453

For the RNase III cleavage assay, 1 µM 200-nt dsRNA was incubated with 1 U 454 RNase III (Invitrogen) and MBP-fusion proteins in reaction buffer according to the 455 456 manufacturer's instructions at 37°C for 30 min. The mature siRNAs processed by RNase III were extracted from the reaction complex by using Trizol reagent (Thermo) 457 458 and subjected to 7 M urea-15% PAGE and then transferred to Hybond-A nylon membrane (GE Healthcare). 459

460

461 Virus infection and plaque assays

At the day of infection, the medium was changed with 2% FBS DMEM and then 462 463 viruses were added in to 293T or Aag2 cells at an MOI of 1. Total RNAs were 464 extracted at 6, 12 and 24 h.p.i., and subjected to Northern blotting and qRT-PCR 465 analysis, respectively. For the rescue experiments, 293T cells were first transfected with the plasmid encoding the indicated proteins, respectively for 24 h.p.t. and treated 466 with Ruxolitinib (10 µM, Selleck) or Enoxacin (100 µM, Selleck) for 1 h, and then 467 infected with viruses. To examine the entry efficiency of SFV into cells, 293T cells 468 were infected with WT or mutant SFV at an MOI of 5, and at 15, 30 and 45 min post 469 470 infection, the viruses remaining in the supernatant and the viral RNAs invaded into 471 cells were examined by plaque assays and qRT-PCR, respectively.

For plaque assays, Vero cells in 12-well plates were infected with 10-fold serial dilution of viruses. Cells were cultured at 37°C for 2 h to allow the adsorption of all the viruses. Then the supernatant was replaced with 1× MEM containing 2% FBS and 1% Penicillin-Streptomycin with isopyknic 1% low-melting-point agarose (Sigma-Aldrich). After incubation at 37°C for 72 h, cells were fixed with 10% formaldehyde and stained with 0.5% crystal violet at 4°C for 2 h. Downloaded from http://jvi.asm.org/ on November 8, 2019 at Univ of Nottingham

478

479 Sucrose density gradient ultracentrifugation and transmission electron
 480 microscopy

The cellular supernatant was filtrated by using the 0.45 μ m filtering membrane and subjected to ultracentrifugation of 150000 g for 3 h at 4°C in a rotor SW28. After Journal of Virology

centrifugation, the precipitate was resuspended in NTE buffer [10 mM Tris-HCl (pH 483 7.5), 120 mM NaCl, 1 mM EDTA] and then put into 10%-60% continuous sucrose 484 485 gradient for ultracentrifugation of 150000 g for 3 h at 4°C in a rotor SW41. The 486 sucrose solution of 30% and 40% containing virions were collected and further 487 subject to ultracentrifugation of 150000 g for 2 h at 4°C in a rotor SW41. In the end, the precipitate was resuspended in 50 µL NTE buffer. 488

For the transmission electron microscopy (TEM), virions were adsorbed to 489 glow-discharged electron microscope grids and negatively stained with the purified 490 491 terephthalic acid according to our standard procedures (50). Samples were imaged on 100KV, HITACHIH-7000FA TEM. 492

493

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631 Figure legends

Figure 1. SFV capsid protein is a potential VSR. (A) S2 cells were co-transfected 632 with a plasmid encoding EGFP (0.1 µg) and dsEGFP (0.3 µg), together with either 633 empty plasmid or a plasmid encoding SFV protein or FHV B2 (FB2) (1 µg for each). 634 635 At 48 h.p.t., total RNAs were extracted and the level of EGFP mRNA was examined via Northern blotting with a DIG-labeled RNA probe targeting 500-720 nt of EGFP 636 ORF region. Rp49 mRNA was used as loading control. (B) The expression of SFV 637 638 proteins was detected by Western blotting. (C) S2 cells were transfected with pMT FHV RNA1 (FHV RNA1) (0.01 µg) or pMT FHV ΔB2 RNA1 (FR1 ΔB2) (0.3 µg) 639 and together with a plasmid encoding SFV capsid or FB2 as indicated above. At 48 640 h.p.t., FHV RNA transcription was induced by incubation with CuSO₄ (0.5 mM). At 641 24 hours after induction, total RNAs were harvested for Northern blot analysis. The 642 643 band between RNA1 and RNA3 was the B2 mRNA transcribed from expression plasmid. The dsRNAs targeting AGO2 (dsAGO2) and Dicer2 (dsDicer2) were used as 644 645 positive controls. (D) 293T cells were co-transfected with a plasmid encoding EGFP 646 $(0.1 \ \mu g)$, EGFP-specific shRNA (shEGFP) $(0.3 \ \mu g)$ together with either empty plasmid or a plasmid encoding SFV capsid protein, nsP2, nsP3 or NB2 (1 µg for each). 647 At 48 h.p.t., total RNAs were extracted and the level of EGFP mRNA was examined 648 via Northern blotting. (E) The expression of SFV nsP2, nsP3 and capsid proteins in 649 293T cells was detected by Western blotting. (F) S2 cells were co-transfected with a 650 651 plasmid encoding EGFP (0.1 μ g), EGFP-dsRNA (dsEGFP) (0.3 μ g) together with either empty plasmid or a plasmid encoding SINV capsid protein, SFV capsid protein, 652 653 or FB2 (1 µg for each). At 48 h.p.t., total RNAs were extracted and the level of EGFP 654 mRNA was examined via Northern blotting. Rp49 mRNA was used as the loading 655 control. (G) 293T cells were co-transfected with a plasmid encoding EGFP (0.1 μ g), EGFP-specific shRNA (shEGFP) (0.3 µg) together with either empty plasmid or a 656 plasmid encoding SINV capsid protein, SFV capsid protein, or NB2 (1 µg for each). 657 At 48 h.p.t., total RNAs were extracted and the level of EGFP mRNA was examined 658 via Northern blotting. GAPDH mRNA was used as the loading control. 659

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Figure 2. SFV capsid suppresses Dicer-mediated siRNA production by 661 662 sequestrating dsRNA. (A) 293T cells were co-transfected with a plasmid encoding EGFP (0.1 μ g), EGFP-specific shRNA (shEGFP) (0.3 μ g), together with either empty 663 664 plasmid or a plasmid encoding SFV capsid protein or NB2 (1 µg for each). At 48 h.p.t., total RNAs were extracted for small Northern blotting with a DIG-labeled RNA 665 oligo probe targeting EGFP siRNA. U6 was used as the loading control. (B) 666 667 SDS-PAGE of purified recombinant SFV capsid. BSA was used as a quantity control. (C) Increasing amount (0-4 µM) of MBP-fusion capsid (MBP-capsid) was incubated 668 with 0.5 µM 200 nt DIG-labeled dsRNA at 25°C for 30 min. Complexes were 669 separated on 1.5% native-TBE agarose gel, transferred to membranes, and then 670 incubated with anti-DIG antibody conjugated with alkaline phosphatase. MBP-FB2 671 672 and MBP were used as the controls. (D) Increasing amount (0-4 μ M) of MBP-capsid was incubated with 1 µM 200-nt DIG-labeled dsRNA at 25°C for 30 min. Then the 673 674 protein-dsRNA complexes were incubated with 1 U RNase III at 37°C for 30 min. 675 The reaction products were subjected to 7 M urea-15% PAGE analysis.

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Figure 3. SFV capsid suppresses siRNA-induced RNAi. (A) 293T cells were 677 678 co-transfected with a plasmid encoding EGFP (0.1 µg), EGFP-specific siRNA (siEGFP) (50 nM), together with either empty plasmid or a plasmid encoding SFV 679 680 capsid protein or NB2 (1 µg for each). At 48 h.p.t., total RNAs were extracted for Northern blotting. GAPDH mRNA was used as the loading control. (B) Increasing 681 682 amount (0-8 µM) of MBP-fusion capsid was incubated with 1 µM DIG-labeled 683 synthetic 22-nt siRNA at 25°C for 30 min. Complexes were separated on 1.5% native-TBE agarose gel, transferred to membranes, and then incubated with anti-DIG 684 685 antibody conjugated with alkaline phosphatase.

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Figure 4. K124/K128 and K139/K142 of SFV capsid are critical for theVSR
activity. (A-B) S2 cells were co-transfected with a plasmid encoding EGFP (0.1 μg)

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capsid deletion mutant as indicated or FB2 (1 µg for each). At 48 h.p.t., total RNAs 690 were extracted for Northern blotting. Rp49 mRNA was used as the loading control. 691 The expression of capsid mutations in S2 cells was detected by Western blotting (B). 692 693 (C-D) The amino acid sequence alignment of alphaviral capsids are as follows: SFV4 (KP699763.1), SFV6 (KT009012.1), SFV-L10 (KP271965.1), SFV-A7 (Z48163.2), 694 695 CHIKV (AOT86261.1), RRV (P08491.3), SINV (AKZ17419.1). S2 cells were 696 co-transfected with a plasmid encoding EGFP (0.1 µg) and dsEGFP (0.3 µg), together 697 with either empty plasmid or the plasmid encoding the indicated single-point mutations of SFV capsid (1 µg for each). At 48 h.p.t., total RNAs were extracted for 698 Northern blotting. The expression of capsid mutations in S2 cells was detected by 699 Western blotting (D). (E-G) Schematic illustration of double-point mutations of SFV 700 701 capsid. S2 cells were co-transfected with a plasmid encoding EGFP (0.1 μ g) and 702 dsEGFP (0.3 µg), together with either empty plasmid or the plasmid encoding the 703 indicated double-point mutations of SFV capsid (1 µg for each). At 48 h.p.t., total 704 RNAs were extracted for Northern blotting (E) and qRT-PCR (G). The expression of capsid mutations in S2 cells was detected by Western blotting (F). (H-I) 293T cells 705 were co-transfected with a plasmid encoding EGFP (0.1 μ g), EGFP-specific shRNA 706 707 (shEGFP) (0.3 μ g) together with either empty plasmid or a plasmid encoding SFV capsid protein or mutations, NB2 (1 µg for each). At 48 h.p.t., total RNAs were 708 709 extracted and the level of EGFP mRNA was examined via Northern blotting. GAPDH mRNA was used as the loading control. The expression of capsid mutations in 293T 710 711 cells was detected by Western blotting (I). (J-K) 293T cells were co-transfected with 712 a plasmid encoding EGFP (0.1 µg), EGFP-specific siRNA (siEGFP) (0.3 µg) together with either empty plasmid or a plasmid encoding capsid_{WT}, capsid_{K124A/K128A}, 713 capsid_{K139A/K142A}, or NB2 (1 µg for each). At 48 h.p.t., total RNAs were extracted and 714 715 the level of EGFP mRNA was examined via Northern blotting. The expression of capsid mutations in 293T cells was detected by Western blotting (K). 716 717

and dsEGFP ($0.3 \mu g$), together with either empty plasmid or a plasmid encoding SFV

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siRNA-binding. MBP-capsid_{K124A/K128A}, 719 and (A-C)MBP-capsid_{WT}, or 720 MBP-capsid_{K139A/K142A} was incubated with 0.5 μ M 200-nt DIG-labeled dsRNA (A), 1 μM 22-nt siRNA (B) or 0.5 μM ssRNA (C) at 25°C for 30 min. The complexes were 721 722 separated on 1.5% native-TBE agarose gel, transferred to membranes, and then incubated with anti-DIG antibody conjugated with alkaline phosphatase. MBP-FB2 723 724 and MBP alone were used as positive and negative control, respectively.

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Figure 6. Construction and recovery of VSR-deficient SFV. (A) SFV genome and 726 727 the mutation sites of K124A/K128A and K139A/K142A. (B) The plaque morphology of SFV_{WT} and SFV_{K124A/K128A}. (C) The virions of SFV_{WT} and SFV_{K124A/K128A} was 728 examined via TEM with magnification times 40000. (D) 293T cells were infected 729 with SFV_{WT} and SFV_{K124A/K128A} at an MOI of 5. At 15, 30 and 45 minutes post 730 731 infection, the viruses remaining in the supernatant and the viral RNAs entered into 732 cells were examined by plaque assays and qRT-PCR, respectively. The RNA levels of SFV_{K124A/K128A} in 293T cells at 15 minutes post infection was defined as 1. All data 733 734 represent means and SD of three independent experiments. n.s., not significant. (E) 293T cells were infected with SFV_{WT} or SFV_{K124A/K128A} (MOI=0.1), respectively. 735 736 Viral titers were measured at the indicated times using standard plaque assay in Vero cells. All data represent means and SD of three independent experiments.*p < 0.05, 737 738 ***p*<0.01 as measured by two-way ANOVA (GraphPad Prism).

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Figure 7. The deficiency of RNAi rescued the defective replication of VSR-deficient SFV. (A) 293T or 293T-NoDice cells were transfected with either empty plasmid or a plasmid encoding NoV B2, mB2 (B2_{R59Q}), SFV capsid or capsid_{K124A/K128A} as indicated. At 24 h.p.t., cells were infected with SFV_{WT} or SFV_{K124A/K128A} at an MOI of 1. At 6, 12, and 24 h.p.i., the levels of SFV genomic RNAs in cells were determined by qRT-PCR, and the level of SFV_{K124A/K128A} RNA in 293T cells at 6 h.p.i. was defined as 1. All data represent means and SD of three 747

independent experiments.*p<0.05, **p<0.01, **p<0.001 as measured by two-way ANOVA (GraphPad Prism). (B) 293T or NoDice 293T cells were transfected with 748 749 either empty plasmid or a plasmid encoding human Dicer (hDicer) as indicated. At 24 h.p.t., cells were treated with Enoxacin (100 μ M) for 1h and then infected with SFV_{WT} 750 751 at an MOI of 1. At 6, 12, and 24 h.p.i., the levels of SFV genomic RNAs in cells were determined by qRT-PCR, and the level of $SFV_{K124A/K128A}$ RNA in 293T cells treated 752 753 with DMSO at 6 h.p.i. was defined as 1. All data represent means and SD of three 754 independent experiments. (C-D) The expression of capsid, capsid_{K124A/K128A}, NoV B2, mB2 or hDicer proteins in 293T or 293T-NoDice cells was detected by Western 755 756 blotting. (E) 293T cells were transfected with SFV capsid or capsid_{K124A/K128A} as indicated, and then infected with SFV_{WT} or $SFV_{K124A/K128A}$ at an MOI of 1. At 24 757 h.p.i., the total RNAs were extracted, the level of SFV genomic and subgeomic RNAs 758 759 were examined via Northern blotting with DIG-labeled RNA probe targeting 760 723-1314 nt of SFV E2 coding region. GAPDH mRNA was used the loading control. 761 (F) 293T-NoDice cells were transfected with either empty plasmid or a plasmid 762 encoding NoV B2, SFV capsid as indicated. At 24 h.p.t., cells were infected with SFV_{K124A/K128A} at an MOI of 1. At 6, 12, and 24 h.p.i., the levels of SFV genomic 763 RNAs in cells were determined by qRT-PCR, and the level of SFV_{K124A/K128A} RNA in 764 293T cells transfected with either empty plasmid at 6 h.p.i. was defined as 1. (G) The 765 expression of SFV capsid, and NoV B2 in 293T-NoDice cells was detected by 766 767 Western blotting. (H) 293T or 293T-NoDice cells were transfected with either empty plasmid or a plasmid encoding NoV B2, mB2 (B2_{R590}), SFV capsid or 768 769 capsid_{K124A/K128A} as indicated. At 24 h.p.t., cells were treated with Ruxolitinib (10 µM) 770 for 1h and then infected with SFV_{WT} or $SFV_{K124A/K128A}$ at an MOI of 1. At 6, 12, and 24 h.p.i., the levels of SFV genomic RNAs in cells were determined by qRT-PCR, 771 and the level of SFV_{K124A/K128A} RNA in 293T cells at 6 h.p.i. was defined as 1. (I) The 772 773 expression of capsid, capsid_{K124A/K128A}, NoV B2, or mB2 in Ruxolitinib treated 293T cells was detected by Western blotting. (J) Aag2 cells were infected with SFV_{WT} or 774 SFV_{K124A/K128A} at an MOI of 1. At 6, 12, and 24 h.p.i., the levels of SFV genomic 775

RNAs in cells were determined by qRT-PCR, and the level of $SFV_{K124A/K128A}$ RNA in MLF cells at 6 h.p.i. was defined as 1. (**K**) Aag2 cells were infected with SFV_{WT} or SFV_{K124A/K128A} at an MOI of 10. At 24 h.p.i., total RNAs were extracted, and the levels of vsiRNAs were examined via Northern blotting with a DIG-labeled RNA probe targeting 1-50 nt of antigenomic SFV RNA. U6 was used a loading control.

781

Figure 8. Model for the suppression of RNAi in mammalian and mosquito cells 782 783 by SFV capsid protein. When SFV entry the host cells, the single-stranded genomic 784 (+)RNAs are used as the templates to produce the negative genomic (-)RNAs. Then the (-)RNAs can be used as the templates to produce more progeny (+)RNAs and 785 786 subgenomic RNAs. The vRI-dsRNAs formed by the 5'-terminal nascent (+)RNAs 787 or/subgenomic RNAs and the (-)RNA template could be recognized and cleaved by 788 Dicer, thus triggering antiviral RNAi. SFV capsid protein can suppress antiviral RNAi 789 by sequestrating dsRNA and siRNA as indicated.

Table 1. The	primers and	oligonucleotides	used in	this study.

	Primers	for	construction	of	SFV	proteins
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Name	Sequence	Restriction enzyme
pAc-v5/His-NS1-anti	GAATTCTATGGCCGCCAAAGTGCATGTTGATAT	EcoR I
pAc-v5/His-NS1-anti	CTCGAGCGTGCACCTGCGTGATACTCTAGTTC	Xho I
pAc-v5/His-NS2-sense	GCGGCCGCATGGGGGTCGTGGAAACACCTCGC	CA Not I
pAc-v5/His-NS2-anti	TCTAGACTACACCCGGCCGTGTGCATGGCTTCT	TC Xbal I
pAc-v5/His-NS3-sense	GAATTCTATGGCACCATCCTACAGAGTTAAGAG	GAGC EcoR I
pAc-v5/His-NS3-anti	TCTAGACTTGCACCCGCGCGGCCTAGTCGCAG	GAC Xbal I
pAc-v5/His-NS4-sense	GAATTCTATGTATATTTTCTCCTCGGACACACTG	GCA EcoR I
pAc-v5/His-NS4-anti	CTCGAGCGACGCACCAATCTAGGACCGCCGTA	GAG Xho I
pAc-v5/His-capsid-sen	se GCGGCCGCATGAATTACATCCCTACGCAAACC	GTTTT Not I
pAc-v5/His-capsid-anti	i CTCGAGCGCCACTCTTCGGACCCCTCGGGGG	TCAC Xho I
pAc-v5/His-E3-sense	GAATTCTATGTCCGCCCGCTGATTACTGCCAT	GT EcoR I
pAc-v5/His-E3-anti	TCTAGAGCGCCGGTGTCTTGTTCCGTTTCGGCA	CG Xbal I
pAc-v5/His-E2-sense	GCGGCCGCATGAGCGTGTCGCAACACTTCAAC	CGTG Not I
pAc-v5/His-E2-anti	CTCGAGCGTGCGTGCGCCCGCGGGGGCGCAGCA	AGAG Xho I
pAc-v5/His-6k-sense	GCGGCCGCCCATGGCTAGTGTGGCAGAGACTA	TGGC Not I
pAc-v5/His-6k-anti	CTCGAGCGAGCTCTGGCGGTTGCCCCGAGGCT	CAGT Xho I
pAc-v5/His-E1-sense	GCGGCCGCATGTACGAACATTCGACAGTAATG	CCGA Not I
pAc-v5/His-E1-anti	CTCGAGCGTCTGCGGAGCCCAATGCAAGTGAC	CACA Xho I
pRK-capsid-sense	GTCGACGATGAATTACATCCCTACGCAAACGT	ITTAC Sal I
pRK-capsid-anti	GCGGCCGCTTACCACTCTTCGGACCCCTCGGG	GGTCA Not I
pMal-c2x-capsid-sense	GAATTCATGAATTACATCCCTACGCAAACGTT	TTAC EcoR I
pMal-c2x-capsid-anti	GTCGACCCACTCTTCGGACCCCTCGGGGGGTCA	ACTC Sal I

Primers for construction of capsid mutants and SFV mutant

Timers for construction of capsic mutants and ST V mutant			
Name	Sequence Res	triction enzyme	
K124A/K128A-sense	TCTTCGAAGTCGCACACGAAGGAGCGGTCACT	GG -	
K124A/K128A-anti	CCAGTGACCGCTCCTTCGTGTGCGACTTCGAAC	GA -	
K139A/K142A-sense	GCCTGGTGGGCGACGCAGTCATGGCACCTGCC	CA -	
K139A/K142A-anti	TGGGCAGGTGCCATGACTGCGTCGCCCACCAG	GC -	
pMD18-T- K124A/K12	8A-sense TCTAGAGACGGACATTGCATCATTC	Xbal I	
pMD18-T- K124A/K12	8A-anti CCTGCAGGAATTCACCCGGTGGGCA	Sbf I	

Primers for qRT-PCR

Name	Sequence	
EGFP-sense	GACAAGCAGAAGAACGGCATC	
EGFP-anti	CGGACTGGGTGCTCAGGTA	
SFV-sense	CCGGAGGACGCACAGAAGTTG	
SFV-anti	TGCGACGGCCACAATCGGAAG	
Human actin-sense	AGAGCTACGAGCTGCCTGAC	
Human actin-anti	AGCACTGTGTTGGCGTACAG	

Aedes aegypti Rp49-sense	GCTATGACAAGCTTGCCCCCA
Aedes aegypti Rp49-sense	TCATCAGCACCTCCAGCT

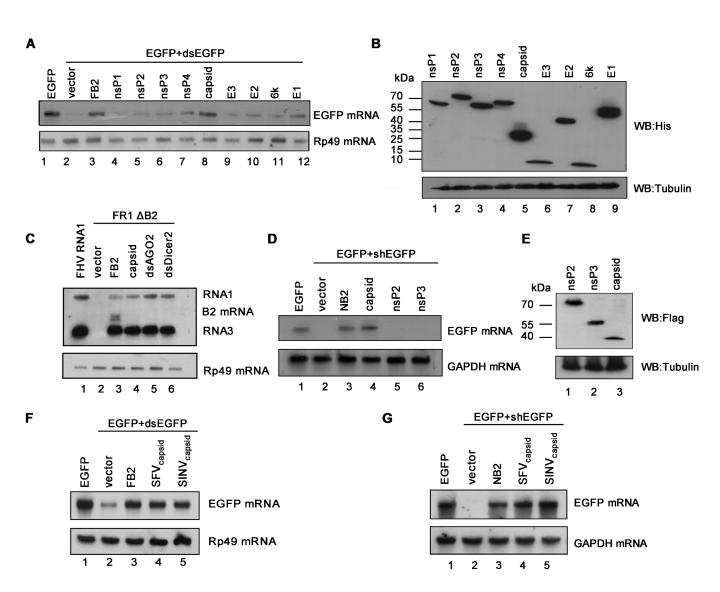
Primers for amplification of templates for *in vitro* transcription dsRNAs and ssRNAs

Name	Sequence
EGFP-ds400-sense	TAATACGACTCACTATAGATGGTGAGCTAGGGCGAGGA
EGFP-ds400-anti	TAATACGACTCACTATAGGCTTGTGCCCCAGGATGTTGC
EGFP-ds200-sense	TAATACGACTCACTATAGATGGTGAGCTAGGGCGAGGA
EGFP-ds200-anti	TAATACGACTCACTATAGGCGGCTGAAGCACTGCACGC
dsAgo2-sense	TAATACGACTCACTATAGATGGGAAAAAAAGATAAGAACAAGC
dsAgo2-anti	TAATACGACTCACTATAGCACCTTGTTGACCTTGTTTAGTCCAC
EGFP-ss200-sense	TAATACGACTCACTATAGATGGTGAGCTAGGGCGAGGA
EGFP-ss200-anti	CTATAGTGAGTCGTATTAGCGGCTGAAGCACTGCACGC

Primers for amplification of templates for in vitro transcription RNA probes

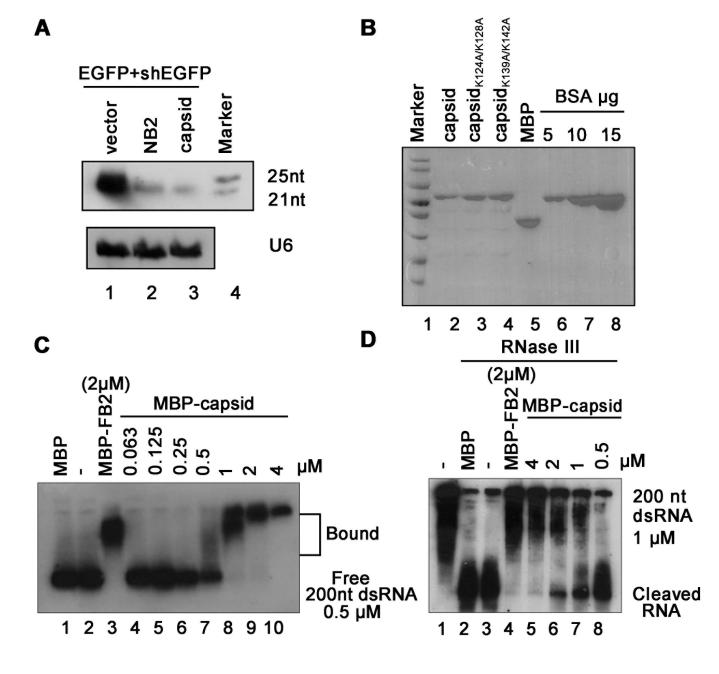
Name	Sequence
EGFP-probe-sense	GATCCGCCACAACATCGAGGACGGC
EGFP-probe-anti	TAATACGACTCACTATAGTTACTTGTACAGCTCGTCCATGCC
S2-Rp49-sense	CCGTGAATACTGTGGTGAAATTGCC
S2-Rp49-sense	TAATACGACTCACTATAGGTTTTTTTTTTCACTTTTAACGTTTCA
GAPDH-probe-sense	GGCGTGATGGCCGCGGGGGCTCTCC
GAPDH-probe-anti	TAATACGACTCACTATAGGCAAAGGTGGAGGAGGGGGGGG
SFV-probe-sense	GCTGCCAAATCAAAACGAACCCTGTCAG
SFV-probe-anti	TAATACGACTCACTATAGGTCTGCGGAGCCCAATGCAAGTGACC
FHV-probe-sense	ATGCCAAGCAAACTCGCGCTAATCCAGGAACTTCCCG
FHV-probe-anti TA	ATACGACTCACTATAGGCCTCTAGGTATGCCACCACGCTGGGTTTCTC
siEGFP-sense GC	UGACCCUGAAGUUCAUCUU
siEGFP-anti GA	UGAACUUCAGGGUCAGCUU
SFV-vsiRNA-probe-	sense
TAATACGACTCAC	TATAGATGGCGGATGTGTGACATACACGACGCCAAAAGATTTTGTTCCA
GCTCCT	
SFV-vsiRNA-probe-	anti
AGGAGCTGGAACAAAATCTTTTGGCGTCGTGTATGTCACACATCCGCCATCTATAGTGAGTC	
GTATTA	

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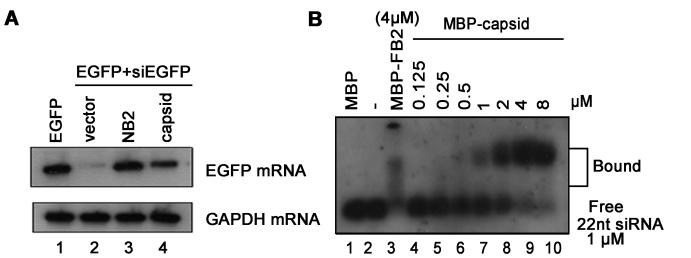


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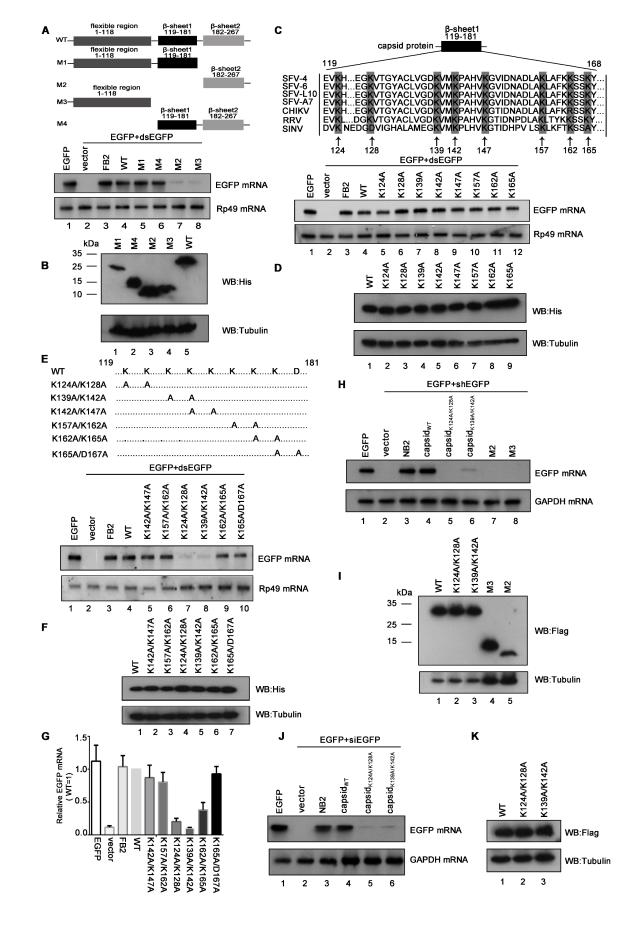


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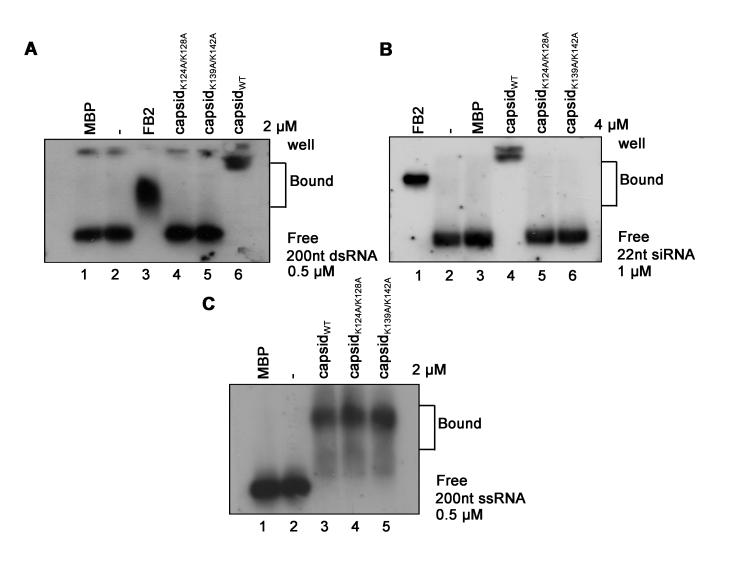




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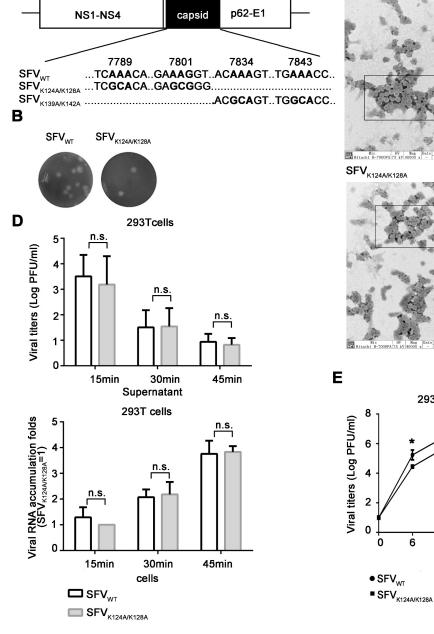


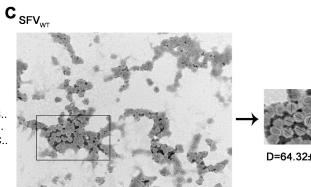
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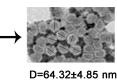


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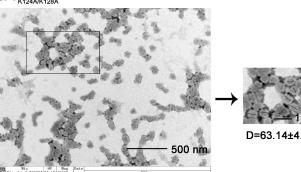
A 5'UTR







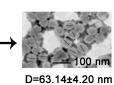
3'UTR

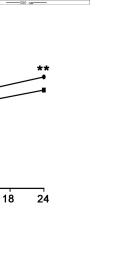


293T cells

12 hpi

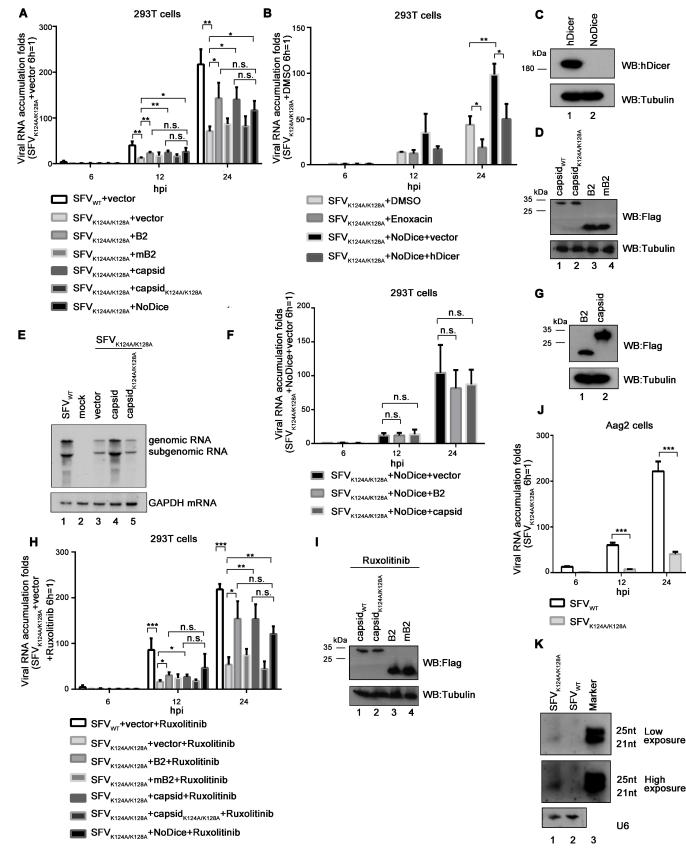
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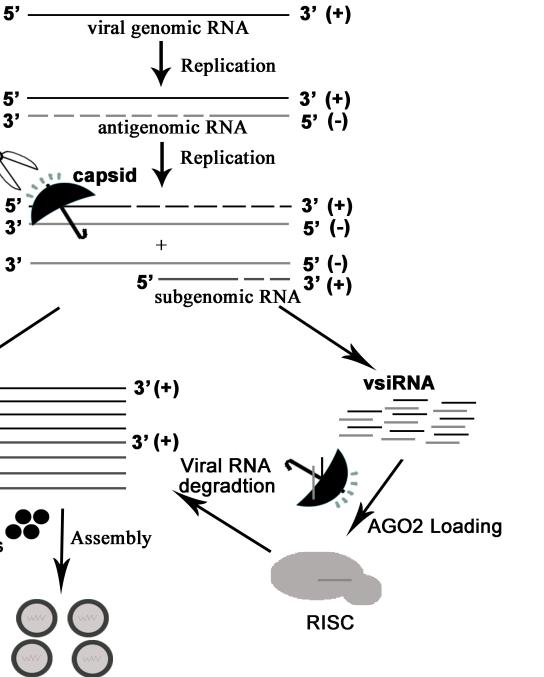
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Dicer

5'

Translation

capsid proteins



5'