A novel interaction between the 5' untranslated region of the Chikungunya virus genome and Musashi RNA binding protein is essential for efficient virus genome replication

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Abstract

Chikungunya virus (CHIKV) is a re-emerging, pathogenic alphavirus that is transmitted to humans by *Aedes spp*. mosquitoes—causing fever and debilitating joint pain, with frequent long-term health implications and high morbidity. The CHIKV replication cycle is poorly understood and specific antiviral therapeutics are lacking. In the current study, we identify host cell Musashi RNA binding protein-2 (MSI-2) as a proviral factor. MSI-2 depletion and small molecule inhibition assays demonstrated that MSI-2 is required for efficient CHIKV genome replication. Depletion of both MSI-2 and MSI-1 homologues was found to synergistically inhibit CHIKV replication, suggesting redundancy in their proviral function. Electromobility shift assay (EMSA) competition studies demonstrated that MSI-2 interacts specifically with an RNA binding motif within the 5' untranslated region (5'UTR) of CHIKV and reverse genetic analysis showed that mutation of the binding motif inhibited genome replication and blocked rescue of mutant virus. For the first time, this study identifies the proviral role of MSI RNA binding proteins in the replication of the CHIKV genome, providing important new insight into mechanisms controlling replication of this significant human pathogen and the potential of a novel therapeutic target.

47 **INTRODUCTION**

48 Chikungunya virus (CHIKV) is transmitted by *Aedes spp.* mosquitos and is an
49 alphavirus of the *Togaviridae* family, which includes other medically relevant
50 alphaviruses such as Semliki Forest virus (SFV), Venezuelan equine encephalitis virus
51 (VEEV), and Sindbis virus (SINV) (1,2). CHIKV was first identified in Tanzania in 1952
52 and symptoms typically include acute febrile symptoms, myalgia, rash and severe
53 arthralgic joint pain, which may persist for months or years (3). CHIKV recently
54 caused epidemic outbreaks across regions of Asia, Africa, the Americas, the Middle
55 East and Southern Europe (3). To date, three phylogenetically distinct lineages of
56 CHIKV have been identified, namely the West African, Asian and the East Central
57 Southern African (ECSA) lineages (4). There remains no clinically approved specific
58 antiviral therapy, due in part to a lack of detailed understanding of the CHIKV
59 replication cycle and its interaction with host cell factors. The first attenuated live
60 CHIKV vaccine (VLA1553) received U.S. FDA approval in November 2023 (5).
61
62 CHIKV is an enveloped, positive-sense single-stranded RNA virus with an ~11.8 Kb
63 genome, containing two open reading frames (ORFs) flanked by 5' and 3'
64 untranslated regions (UTRs) (**Fig. 1A**). The 5' UTR is ~ 76 nts in length and is capped
65 by 5' type-0 N-7-methylguanosine. The upstream ORF (ORF-1) encodes the viral non-
66 structural proteins 1-4 (nsP1-4), which are translated directly from the genomic RNA
67 as a single polyprotein that is subsequently proteolytically cleaved into the four
68 mature proteins (6). Through analogy with other alphaviruses, proteolytic cleavage
69 of nsP1-4 in *cis* by nsP2 releases nsP4 which, as the RNA-dependent RNA polymerase

70 (RdRp), initiates synthesis of the minus-strand intermediate RNA. Subsequent
71 proteolytic cleavage of the remaining nsP123 polyprotein initiates replication of
72 genomic and sub-genomic (29S) RNAs from the minus-strand template (7). The
73 downstream ORF (ORF-2) encodes the structural polyprotein that is processed into
74 the capsid and envelope proteins, E3, E2, 6K, and E1.
75
76 While negative strand replication is initiated at the 3' end of the virus genome, as
77 with many positive single stranded RNA viruses, regulatory elements and
78 interactions within the 5' end of the CHIKV genome are required for its initiation and
79 regulation (8). We and others have previously demonstrated that such regulatory
80 elements include functional RNA secondary structures and higher order interactions
81 within the CHIKV 5'UTR and adjacent upstream region of ORF-1 (Fig 1). It has been
82 speculated that these RNA elements may regulate template specificity and temporal
83 control of switching, between CHIKV translation and genome replication (9,10).
84
85 In a previously published study, we demonstrated by SHAPE mapping and reverse
86 genetic analysis that the CHIKV 5'UTR and adjacent ORF-1 coding region is highly
87 structured (10). However, the study also highlighted a single-stranded region (nts 63-
88 69) exhibiting a high degree of nucleotide conservation that was located
89 immediately between two RNA structures (Supplementary data 1), which we
90 demonstrated were essential for initiation of CHIKV genome replication (Fig 1B).
91 Interestingly, *in silico* analysis noted that nts $_{63}$AUUAAU$_{68}$ were closely homologous
92 to the Musashi RNA binding protein (MSI) consensus binding sequence ((G/A)U$_{1-}$
93 $_{3}$AGU). MSI are highly conserved RNA binding proteins, containing two highly

94 conserved tandem RNA recognition motifs (RNP-1 and RNP-2), that interact with
95 RNA via the same consensus binding motif. Two MSI homologues have been
96 identified, MSI-1 and MSI-2, that share over 90% homology in their RNA-binding
97 domains and a high degree of functional complementarity and redundancy (11,12).
98 MSI have key roles in post-transcriptional regulation of genes involved in
99 development, cell cycle regulation and maintenance of adult neural stem/progenitor
100 cells (13). A recent study demonstrated that MSI-1 promotes Zika Virus (ZIKV)
101 genome replication in neurons, via interaction with the consensus binding site within
102 the viral 3'UTR (14).
103
104 In the current study, we demonstrate through a range of infectious clone and sub-
105 genomic replicon reporter assays that MSI-2 is required for efficient CHIKV genome
106 replication. A small molecule inhibitor of MSI-1 and MSI-2 reduced infectious virus
107 production by inhibition of CHIKV genome replication. Similarly, MSI-2 silencing by
108 shRNA and siRNA inhibited both infectious virus production and genome replication,
109 while co-silencing of both MSI-2 and MSI-1 exhibited a synergistic effect on inhibition
110 of CHIKV replication. Reverse genetic analysis, in which the putative MSI binding site
111 within the CHIKV 5'UTR was mutated, completely prevented rescue of mutant virus
112 and inhibited CHIKV genome replication. Biochemical MSI-2 binding analysis by
113 competition electromobility shift assay (EMSA) demonstrated that recombinantly
114 expressed MSI-2 specifically interacts with the 5' region of the CHIKV genome and
115 that this interaction is inhibited by mutation of the putative MSI 5'UTR binding site.
116 These findings demonstrate for the first time that interaction between MSI and a
117 binding site within the 5'UTR are required for replication of the CHIKV genome.

118

119 **Materials and Methods**

120

121 **Cell culture**

122 Human Rhabdomyosarcoma (RD), Human hepatoma (Huh7), Baby Hamster Kidney
123 (BHK) and Human Embryonic Kidney (HEK) 293 were grown in Dulbecco's modified
124 eagle's medium (DMEM; Sigma) containing 10% foetal bovine serum (FBS; PAA), 1x
125 penicillin-streptomycin (Sigma), 25mM HEPES in 0.85% NaCl (Lonza) and 1% non-
126 essential amino acids mixture (NEAA; Lonza). Cells were harvested using
127 trypsin/EDTA (Sigma) and maintained at 37°C in 5% CO_2.

128

129 **CHIKV cDNA plasmid**

130 The infectious CHIKV (ICRES), CHIKV Dual-luciferase sub-genomic replicon (CHIKV-
131 SGR) and *trans*-complementation (pCHIKV-nsP1234 and pCHIK-Fluc/Gluc) assay were
132 derived from the CHIKV ECSA strain, isolate LR2006 OPY1 (accession number
133 DQ443544)(15). In CHIKV-SGR the second ORF was replaced by a firefly luciferase
134 gene and a *Renilla* luciferase gene was fused within nsP3 (16). The *trans*-
135 complementation assay utilised a CMV codon optimised plasmid to supply the CHIKV
136 replicase in *trans* (pCHIKV-nsP1234) and a Pol II expressed reporter plasmid (pCHIKV-
137 FLuc/GLuc), in which the majority of ORF-1 (downstream of nt 320) was replaced by
138 a firefly luciferase gene and all of ORF-2 by a Gaussia Luciferase gene (17). Plasmid
139 cDNA was purified using GeneJET Plasmid Maxiprep kits (Thermo Fisher Scientific)
140 according to the manufacturer's instructions.

141

142 ***In vitro* RNA transcription**

143 To generate CHIKV-SGR and infectious CHIKV RNA, 2 µg of cDNA was linearised with
144 Not-I HF and used as a template for transcription of 5' [m7G(5')ppp(5')G] capped
145 (m7G capped) RNA from an upstream SP6 promoter, using an SP6 mMessage
146 mMachine kit, according to the manufacturer's instructions (Life Technologies).
147 Uncapped CHIKV 1–337 RNA, used 1 µg of CHIKV 1–337 PCR DNA as a template for *in*
148 *vitro* transcription using the SP6-Scribe™ standard RNA IVT kit, according to the
149 manufacturer's instructions (Lucigen). In all cases, following DNase I treatment, RNA
150 was purified by LiCl precipitation and analysed by denaturing agarose gel
151 electrophoresis.
152

153 **Virus production**

154 1×10^6 BHK cells were trypsinised and resuspended in 400 µl ice-cold DEPC-PBS. Cells
155 were then electroporated with 1 µg 5'-capped CHIKV ICRES *in vitro* transcribed RNA
156 in a 4 mm electro-cuvette, with a single square wave pulse at 260 V for 25 ms using a
157 Bio-Rad electroporator, before seeding into a T175 flask in 20 ml DMEM. After 24 h,
158 supernatant was aspirated and virus titre measured by plaque assay on BHK cells.
159

160 **CHIKV quantification by plaque assay**

161 BHK cells were seeded at 1×10^5 cells per well in 12-well plates and maintained
162 overnight in 1 ml DMEM. The following day monolayers were washed with PBS,
163 infected with 10-fold serial dilutions of CHIKV transfection supernatant and

164 maintained at 37°C. At 1 hour post infection (h.p.i.) monolayers were washed with
165 PBS and covered with a 0.8% methylcellulose DMEM P/S overlay. Monolayers were
166 fixed and stained at 48 h.p.i. (5% paraformaldehyde and 0.25% crystal violet
167 respectively), plaques counted and virus titres expressed in plaque-forming units per
168 ml (PFU/ml).
169
170 **Ro 08-2750 cell viability assay**
171 RD cells were seeded in 96-well plate at $8x10^4$ cells/well and maintained for 24 h.
172 Monolayers were then treated with increasing doses (0, 0.5, 1, 3, 5, 7, 10, 20 µM) of
173 the MSI small molecule inhibitor Ro 08-2750 (TOCRIS) dissolved in DMSO. After 24 h
174 media/inhibitor was aspirated and replaced with 20 µl of 5 mg/mL 3-(4,5-
175 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) and incubated
176 at 37°C in 5% CO_2 for 3 h. After incubation, MTT solution was replaced with 100 µl
177 DMSO and the plate shaken at 60 rpm for 5 min. Absorbance at 570 nm was
178 determined using an Infinite F50 microplate reader (Tecan) and expressed as a
179 percentage of DMSO control cells. 5 µM Ro 08-2750 was estimated to be the
180 maximum non-toxic dose in in Huh7 cells and was used for all further assays.
181
182 **Infectious CHIKV Ro 08-2750 inhibition assays**
183 RD cells were seeded in 12-well plates at $1x10^5$ cells/well and incubated overnight in
184 the presence of Ro 08-2750. Monolayers were then infected with CHIKV at MOI=0.1
185 and adsorbed to the cells for 1 h at 37°C before aspirating and maintaining in the
186 presence of Ro 08-2750 for 24 h. Supernatants was then collected and infectious
187 virus production measured by plaque assay.

188

189 **siRNA depletion of MSI-1 and MSi-2**

190 RD or Huh7 cells were seeded at $1x10^5$ cell/well in 12-well plates in antibiotic-free
191 medium. After 24 h, cells were washed with PBS and incubated with 1x Opti-MEM +
192 GlutaMAX (Gibco) for 20 min at 37°C/5% CO_2. For each well, 50 pmol MSI-1 (sc-
193 106836; Santa Cruz) and/or MSI-2 siRNA (sc-75834; Santa Cruz) were mixed with 100
194 µl Opti-MEM and incubated at room temperature for 1min. No siRNAs were added
195 to mock samples and 50 pmol of scrambled siRNA (SI03650318; QIAGEN) was used
196 as a negative control. In parallel, 3 µl Lipofectamine RNAiMAX (Invitrogen) was
197 mixed with 100 µl Opti-MEM and incubated at room temperature for 1 min. The
198 siRNA and Lipofectamine RNAiMAX were mixed and incubated at room temperature
199 for 5 min before adding to the cells. After 24 h, cells were either lysed to confirm
200 MSI-1 or MSi-2 depletion by western blot or used for subsequent CHIKV infection
201 assays.

202

203 **shRNA depletion of MSI-1 and MSi-2**

204 Human embryonic kidney 293 (HEK 293T) cells were plated in antibiotic-free DMEM
205 in 6-well plates and maintained until confluency reached ~80%. For each
206 transfection, in a single tube 300 µL OptiMEM was mixed with 1 µg packaging
207 plasmid p8.91, 1 µg envelope plasmid pMDG encoding VSV-G protein and 1.5 µg
208 lentiviral vector plasmid encoding shRNA to MSI-2 (sc-75834-SH; Santa Cruz
209 Biotechnology). A lentiviral vector plasmid encoding scrambled shRNA sequences
210 with no cellular targets was used as the negative control (sc-108060; Santa Cruz
211 Biotechnology). In another tube, 300 µL of OptiMEM was mixed with 5 µL

212 lipofectamine 2000 (Invitrogen). Both tubes were gently mixed by flicking, incubated
213 at room temperature for 5 min, mixed together and incubated for 20 min at room
214 temperature. The antibiotic-free DMEM was aspirated and the monolayers washed
215 once with PBS. 800 µL Opti-MEM was added to each well before dropwise addition
216 of the lentiviral plasmids/shRNA mixture. After maintenance at 37°C for 6 h the
217 media was changed to antibiotic-free DMEM. 48 hpt the lentivirus containing
218 supernatant was harvested and filtered through a 0.45 µm filter.
219
220 RD cells were seeded at $1x10^5$ cells/well the day prior to transduction. 1 mL of the
221 lentivirus supernatant and polybrene (MERCK) were added to each well and
222 incubated for 6 h before aspirating and replacing with antibiotic free DMEM. After
223 72 h media was replaced with DMEM containing 2.5 µg/ml puromycin, in which the
224 cells were then maintained. The efficiency of the shRNA MSI-2 depletion was
225 confirmed by western blot.
226
227 **Strand-specific Quantification of CHIKV RNA**

228 Total RNA was extracted from infected cells using TRI Reagent Solution (Applied
229 Biosystems) according to the manufacturer's instructions. Strand-specific RT-qPCR
230 (ssRT-qPCR) was performed as previously described (18). Briefly, 500 ng of RNA was
231 reverse-transcribed with gene specific primers (Supplementary data 2) using the
232 SCRIPT cDNA Synthesis Kit (Jena Bioscience) according to the manufacturer's
233 protocol. 100 ng of strand-specific cDNA was used as template for the quantitative
234 PCR performed with the qPCRBIO SyGreen Blue Mix Lo-ROX (PCR Biosystems), with

235 gene specific primers amplifying a 94 bp region of the CHIKV nsP1-encoding
236 sequence using the following PCR program: 95°C for 2 mins, 40 x (95°C for 5 sec,
237 60°C for 30 sec), dissociation curve 60°C-95°C as pre-defined by the Mx3005P
238 thermal cycler (Agilent technologies). *In vitro* transcribed CHIKV ICRES RNA was
239 reverse transcribed and a cDNA dilution series employed as a standard to quantify
240 copy numbers in the respective samples.

241

242 **CHIKV Sub-genomic replicon assays**

243 For analysis of Ro 08-2750 inhibition, RD cells were seeded in 24 well plates at
244 5×10^4 cells per well and maintained overnight in the presence of Ro 08-2750.
245 Monolayers were washed once in PBS before addition of 400 µl opti-Mem reduced-
246 serum media and 100 µl transfection media. Transfection media was prepared
247 according to the manufacturer's instructions, using 1 µl Lipofectamine 2000
248 (Invitrogen), 250 ng of CHIKV-SGR RNA and appropriate concentrations of Ro 08-
249 2750, before being made up to 100 µl using opti-Mem. Monolayers were maintained
250 for 6 hpt before the media was aspirated and replaced with complete DMEM/Ro 08-
251 2750. At 8 and 24 hpt monolayers were lysed with 100 µl 1 x passive lysis buffer
252 according to the manufacturer’s instructions (Promega), stored at −80°C and
253 analysed using Dual-luciferase substrate (Promega) in a FLUOstar Optima
254 luminometer (BMG labTech). For shRNA MSI-2 depleted cell lines the same method
255 was followed, with the exclusion of Ro 08-2750.

256

257 ***Trans*-complementation Assay**

258 The *trans*-complementation assay was performed as previously described (19).
259 Briefly, for analysis of Ro 08-2750 inhibition, RD cells were seeded in 12 well plates at
260 5×10^4 cells per well and maintained overnight in the presence of Ro 08-2750.
261 Monolayers were then co-transfected with 1 µg each of the pCHIKV-nsP1234 and
262 pCHIKV-FLuc/GLuc using Lipofectamine 2000, as described previously, and assayed
263 at 8 and 24 hpt. For shRNA MSI-2 depleted cell lines the same method was followed,
264 with the exclusion of Ro 08-2750.

265

266 **Expression and purification of recombinant MSI-2 RNA binding domains**

267 The MSI-2 RNA binding domain expressing plasmid pET-22HT-MSI-2 (amino acid 8-
268 193 and referred to hereafter as MSI-2 8-193) was a kind gift from Prof S. Ryder
269 (Addgene plasmid # 60356; http://n2t.net/addgene:60356; RRID: Addgene_60356)
270 (20). The plasmid was transformed into BL21 (DE3) competent cells following the
271 manufacturer's protocol (NEB), a single colony was inoculated into 10 mL LB
272 ampicillin (10 mg/ml) and incubated overnight at 37°C before inoculating 1000 ml 1
273 LB (ampicillin (10 mg/ml) and adding 100 µM Isopropyl β-D-1-thiogalactopyranoside
274 (IPTG; Thermo Fisher Scientific) when the optical density reached 0.8. The culture
275 was then incubated overnight at 18°C in an orbital shaker before centrifugation to
276 pellet the bacteria. The pellet was resuspended in lysis buffer (20U/mL DNase I,
277 0.6U/mL RNase A, 1mg/mL lysozyme and 1x protease inhibitor) and lysed on ice for
278 30 min. Following sonication, the suspension was centrifuged twice at 2000 xg for 1
279 hour at 4°C. The was filtered through a 0.45 µm filter and His-MSI-2 was purified
280 with HisTrap™ FF column (Cytiva) using the Econo Gradient Pump (BIORAD)

281 according to the manufacturer's protocol and overnight dialysis. The purified protein
282 was quantified and the purity and identity assayed by Coomassie SDS-PAGE and
283 western blot (Supplementary data 3).
284
285 After desalting using PD-10 desalting columns (Cytiva), His-MSI-2 protein was further
286 purified by ion exchange chromatography. Following the manufacturer's
287 instructions, the column was equilibrated with wash buffer (50 mM MES, 10 mM
288 NaCl, pH 5.6, degassed) and the protein eluted in the same buffer. Ion exchange
289 chromatography was performed using HiTrap SP HP cation exchange
290 chromatography column (Cytiva) following the manufacturer's instructions and
291 eluted in degassed 50mM MES, 1M NaCl, pH 5.6. The eluted protein fractions were
292 analysed by Coomassie SDS-PAGE and western blot (Supplementary data 3).
293
294 **^{32}P RNA end labelling**
295 *In vitro* transcribed CHIKV RNA (nt's 1-337) was 5' dephosphorylated using Quick CIP
296 according to the manufacturer's instructions (NEB) before purification using an RNA
297 Clean & Concentrator column (Zymo Research). 20 pmoles of dephosphorylated RNA
298 was combined with 2 µl 10 x T4 Polynucleotide Kinase buffer (NEB), 3 µl ATP(γ-^{32}P)
299 10 mCi/ml, 1 µl T4 Polynucleotide Kinase (NEB) and nuclease free H_2O to a final
300 volume of 20 µl, incubated at 37°C for 60 min, 65°C for 20 min, purified using an RNA
301 Clean & Concentrator Kit (Zymo Research) and resuspended in RNase-free H_2O.
302
303 **Electromobility shift assay (EMSA)**

304 RNA was incubated at 95°C for 2 min and on ice for 2 min before 3.3 x RNA folding
305 buffer (100 mM HEPES pH 8.0, 100 mM NaCl and 10 mM MgCl2) and RNasin Plus
306 RNase Inhibitor (Promega) was added to a final volume of 10 µl and incubated for
307 20 min at 37°C. MSI-2 protein was combined with 3.3 x RNA folding buffer, 0.5 x TE,
308 100% glycerol, RNasin Plus RNase Inhibitor (Promega) and 5 µg yeast tRNA as non-
309 specific competitor and incubated at 37°C for 2 min before adding to the RNA
310 mixture and further incubation at 37°C for 15 min. Finally, samples were analysed by
311 native PAGE gel electrophoresis at 135 V for ~2 h. The gel was fixed for 30 min and
312 dried with gel dryer (BIORAD), exposed onto Hyperfilm™ ECL™ (Merck) and
313 visualised using a Xograph Film Processor. Band shifts in the competition EMSAs
314 were quantified by measuring the density of the RNA/Protein complex band (as
315 indicated by square brackets) normalized to the density of the whole lane and
316 expressed as % of the normalized RNA/Protein complex band in lane 1:0.

317 **Western blotting**

318 Following infection and incubation, monolayers were lysed in IP lysis buffer
319 (Promega) and incubated at room temperature for 30 min. Protein concentration
320 was quantified using a Pierce™ BCA Protein Assay Kit (Thermo scientific) according to
321 the manufacturer's instructions. Equal amounts of protein lysate were analysed by
322 SDS-PAGE. Protein was transferred onto an Immobilon-FL PVFD transfer membrane
323 (MERCK) using a TE77X semi-dry transfer (Hoefer) at 15 V for 60 min. Membranes
324 were blocked using diluted Odyssey® Blocking Buffer in PBS (LI-COR) for 30 min and
325 probed with primary antibodies against β-actin (1:10,000, mouse monoclonal, Sigma-
326 Aldrich A1978), MSI-2 (1:1000 rabbit monoclonal, Abcam ab76148) and MSI-1

327 (1:5000, Abcam ab21628) in diluted Odyssey® Blocking Buffer in PBS (LI-COR)
328 overnight at 4°C. After overnight incubation, primary antibody was removed and
329 membranes washed 3 times using PBS. Membranes were stained with secondary
330 antibodies (IRDye® 800CW Donkey anti-Mouse; IRDye® 680LT Donkey anti-Rabbit; Li-
331 Cor) for 1 h at room temperature, washed 3 times using 1 x PBS, dried and then
332 imaged using an Odyssey® Fc Imaging System (Li-Cor).
333

334 **Statistical analysis**

335 Statistical analysis was carried out using two-tailed Students Test comparisons on
336 GraphPad Prism version 8.4.0. *P* values of ≤0.05 (*), ≤0.01 (**), ≤0.001 (***) were
337 used to represent degrees of significance between each drug
338 treatment/silencing/mutant to wild-type assay.
339

340 **RESULTS**

341

342 **MSI-2 binds specifically to the predicted $_{63}$AUUAAU$_{68}$ 5'UTR MSI binding site in the**
343 **CHIKV 5'UTR**

344 In order to examine the potential for direct interaction between the RNA binding
345 domains of MSI-2 and nucleotides $_{63}$AUUAAU$_{68}$ within the CHIKV 5'UTR, which had
346 close sequence homology to the (G/A)U$_{1-3}$AGU MSI consensus nucleotide binding
347 motif, we used native EMSA (21). $_{63}$AUUAAU$_{68}$ is located in a single-stranded region
348 of the 5' UTR, 9 nts upstream of the AUG start codon and between two RNA
349 structures that are essential for CHIKV genome replication (Fig 1B)(10). The first

350 330nts of the CHIKV genome (RNA-330), representing the 5'UTR and adjacent
351 upstream region of ORF1, was *in vitro* transcribed, 5' end radiolabelled with ATP-[γ-
352 32P] and incubated at 37°C - conditions under which correct folding of the functional
353 RNA-330 structure was previously validated (10). Following incubation with
354 increasing concentrations of recombinantly expressed and purified MSI-2 8-193 in
355 the presence of unlabelled tRNA, reaction products were separated by native PAGE
356 and analysed by autoradiography (Fig 2A). Relative to unbound RNA-330, the
357 presence of MSI-2 8-193 retarded RNA-330 migration during native PAGE, consistent
358 with the formation of an RNA-330/MSI-2 8-193 complex, the increasing size of the
359 retarded band potentially indicating MSI-2 multimerization. In order to investigate
360 the specificity of the observed interaction, a fixed molar ratio of RNA-330$^{\gamma\text{-}32P}$ and
361 MSI-2 8-193 was incubated with increasing concentrations of competitor unlabelled
362 RNA-330 (Fig 2B). Increasing concentrations of unlabelled RNA-330 reduced the
363 formation of the RNA-330$^{\gamma\text{-}32P}$/MSI-2 8-193 complex, with a corresponding increase
364 in unbound RNA-330$^{\gamma\text{-}32P}$.
365
366 To further investigate the specificity and location of the interaction, the putative
367 MSI-2 binding site $_{63}$AUUAAU$_{68}$ was mutated to $_{63}$CAACUU$_{68}$ (henceforth termed
368 $_{63}$CAACUU$_{68}$-mut), which was predicted *by in silico* analysis not to affect local RNA
369 structure (Supplementary data 4A and B). EMSA competition, with unlabelled
370 $_{63}$CAACUU$_{68}$-mut RNA-330, was less efficient at competing for MSI-2 8-193 binding
371 than wild-type CHIKV RNA (Fig 2C and D). These EMSA results are consistent with an
372 interaction between MSI-2 and the upstream 1-330 nt region of the CHIKV genome.
373 Furthermore, reduced competition with mutant $_{63}$CAACUU$_{68}$-mut compared with

374 wild type, was consistent with a specific interaction, in which nts $_{63}$AUUAAU$_{68}$

375 function as an MSI binding motif.

376

377

378 **Replication of CHIKV infectious virus and sub-genomic replicon was restricted by**

379 **MSI small molecule inhibitor Ro 08-2750:**

380 In order to investigate the potential role of MSI on CHIKV replication, we first

381 assessed the effect of a well-characterised MSI small molecule inhibitor, Ro 08-2750,

382 on CHIKV productive replication in RD cells using both CHIKV infectious virus and a

383 sub-genomic replicon (SGR) assay (Fig 3A). A number of studies have demonstrated

384 that Ro interacts with the MSI RNA binding domains and acts as a competitive

385 inhibitor for its RNA binding activity and subsequent MSI functions within the cell

386 (22,23) (24). In the current study Ro 08-2750 was used at a maximum non-toxic dose

387 as determined by MTT assay (Supplementary data 5).

388

389 To assay the effect of Ro 08-2750 on early and late stages of CHIKV replication,

390 productive CHIKV infection was measured at 8 and 24 hpi. Pre-treatment and

391 incubation in the presence Ro significantly inhibited infectious CHIKV replication by

392 ~10-fold, relative to DMSO treated negative controls (Fig 3B). In order to investigate

393 the effect of Ro 08-2750 on specific stages of CHIKV replication we used an SGR

394 construct, in which ORF-2 was replaced by a firefly luciferase (FLuc) gene and a

395 *Renilla* luciferase (Rluc) gene was fused in-frame with ORF-1 nsP3 (Fig 3A)(10). The

396 SGR assay enabled us to measure the effect of Ro 08-2750 on CHIKV genome

397 replication and translation, in isolation of other stages of virus infection, such as
398 entry or egress. SGR ORF-1 and ORF-2 expression was significantly inhibited at both 8
399 and 24 hpt, relative to DMSO treated negative controls, indicating that Ro 08-2750
400 inhibited CHIKV replication at the level of virus genome replication or translation (Fig
401 3C).
402
403 In order to investigate the possibility that Ro 08-2750 specifically inhibited ORF-1
404 translation, we used a replication incompetent SGR, incorporating an nsP4 GDD>GAA
405 substitution in the RdRp active site (CHIKV-SGR (GDD>GAA)). Such an approach
406 enabled the efficiency of ORF-1 translation to be measured by *Renilla* luciferase
407 expression, in the presence and absence of Ro 08-2750, under conditions in which
408 genome replication could not occur. Translation of input SGR 5'capped transcripts
409 was measured at 6 hpt in RD cells (Fig 3E). No significant difference in level of
410 translation was observed between the CHIKV-SGR (GDD>GAA) in the presence or
411 absence of Ro 08-2750. These results indicate that Ro 08-2750 did not influence ORF-
412 1 translation but inhibited SGR genome replication.
413
414 **Ro inhibited CHIKV *trans*-complementation assay:**
415 Inhibition of SGR replication demonstrated that Ro was inhibiting CHIKV replication
416 at the level of virus genome replication or translation. In order to dissect this further,
417 we utilised a *trans*-complementation assay, that enabled the measurement of CHIKV
418 genome replication in isolation of virus translation (Fig 4A)(25). The *trans*-
419 complementation system utilised a CHIKV replicase-expressing plasmid (pCHIKV-
420 nsP1234). pCHIKV-nsP1234 , expresses the virus nsPs from a CMV promoter and is

421 codon optimised to disrupt RNA sequence motifs, such as protein binding signals,
422 and the formation of wild-type CHIKV RNA structure. The expressed nsPs replicate a
423 CHIKV reporter construct (pCHIKV-FLuc/GLuc), in which the majority of ORF-1 is
424 replaced by an FLuc gene and ORF-2 by the Gaussia luciferase (GLuc) gene. The effect
425 of Ro on pCHIKV-FLuc/GLuc expression was measured at 8 and 24 hpt in cells pre-
426 treated with and maintained in the presence of Ro. Both ORF-1 and ORF-2 expression
427 was observed to be significantly inhibited relative to the DMSO treated negative
428 controls, indicating that Ro was specifically inhibiting CHIKV genome replication.
429
430 **MSI-2 shRNA silencing inhibits infectious CHIKV replication**
431 In order to confirm that Ro induced inhibition of CHIKV genome replication was due
432 to specific inhibition of MSI, rather than an unrecognised off target effect of Ro, we
433 next investigated CHIKV replication following shRNA silencing of MSI expression.
434 Analysis of RD cell total protein extract by western blot, demonstrated that MSI-2
435 was strongly expressed in RD cells while MSI-1 was expressed at a very low level
436 (Supplementary data 6). Consequently, we initially investigated the effect of specific
437 MSI-2 shRNA silencing on CHIKV replication. RD cells were transduced with lentiviral
438 vectors encoding shRNA against human MSI-2 and successful silencing was
439 confirmed by western blot (Fig 5A). Negative control cells were treated with either
440 transfection reagent only (mock) or non-specific scrambled shRNA. Following
441 infection of MSI-2 depleted and negative control cells, productive CHIKV infection
442 was measured by plaque assay at 8 and 24 hpi (Fig 5B). Strand specific qRT-PCR was
443 also used to measure levels of genomic (positive strand) and replication
444 intermediate (negative strand) CHIKV RNA at both time points (Fig 5C and D). As

445 observed previously following treatment with Ro, relative to the scrambled shRNA
446 control productive CHIKV replication was significantly inhibited following shRNA MSI-
447 2 silencing (Fig 5B). Similarly, strand specific qRT-PCR demonstrated that both
448 genomic and replication intermediate CHIKV RNA levels were significantly inhibited
449 (Fig 5C and D). These results are consistent with CHIKV requiring MSI-2 for efficient
450 genome replication.
451
452 **MSI-2 is required for efficient CHIKV genome replication**
453 In order to confirm that MSI-2 silencing had the same effect on CHIKV genome
454 replication as Ro small molecule inhibition, we repeated analysis with the SGR and
455 *trans*-complementation systems, following shRNA silencing of MSI-2. As previously
456 described, MSI-2 shRNA silenced RD cells were transfected with the SGR system and
457 ORF-1 and ORF-2 expression measured by RLuc and FLuc expression at 8 and 24 hpt
458 (Fig 6A and B). Expression of both ORF-1 and ORF-2 was significantly inhibited at
459 both 8 and 24 hpt, consistent with an MSI-2 requirement for CHIKV genome
460 replication or translation. Analysis at the same time points using the *trans*-
461 complementation system, following shRNA MSI-2 silencing and measuring ORF1 and
462 ORF2 expression, confirmed significant inhibition of CHIKV genome replication when
463 measured in isolation of the effects of other stages of the virus replication cycle (Fig
464 6C and D)
465
466 **Both MSI-2 and MSI-1 have a proviral effect on CHIKV genome replication:**
467 While results clearly demonstrated that MSI-2 was required for efficient CHIKV
468 replication, it remained unclear if MSI homologue MSI-1 was also agonistic for CHIKV

469 replication. As previously described, western blot analysis showed that MSI-1 was
470 not highly expressed in RD cells. Consequently, we analysed MSI homologue
471 redundancy in Huh7 human hepatoma cells. MSI-1 and MSI-2 homologues are
472 strongly expressed in Huh7 cells (Supplementary data 6) and they are highly
473 permissive for CHIKV replication (26). Following individual and combined siRNA
474 silencing of MSI-1 and MSI-2 in Huh7 cells (Supplementary data 7), CHIKV replication
475 was assayed by plaque assay and ssRT-qPCR at 8 and 24 hpi (Fig 7A, B and C). As was
476 observed for RD cells, MSI-2 silencing significantly inhibited CHIKV replication,
477 measured by both plaque assay and strand specific ssRT-qPCR. Interestingly, it was
478 observed that MSI-1 silencing in Huh7 cells had a similar significant inhibitory effect
479 and that siRNA co-silencing of both MSI-1 and MSI-2 had a synergistic effect on
480 inhibition of CHIKV replication. In concordance with previous results, siRNA silencing
481 of MSI-2 in RD cells significantly inhibited CHIKV replication, by comparable levels
482 observed following shRNA silencing and inhibition by Ro. Consistent with low MSI-1
483 expression in RD cells, siRNA co-silencing of both MSI-1 and MSI-2 did not
484 significantly increase levels of CHIKV inhibition (Fig 7D, E and F).
485
486 **Reverse genetic analysis of $_{63}$AUUAAU$_{68}$ MSI binding site**
487 In order to further investigate the role of the $_{63}$AUUAAU$_{68}$ MSI binding motif in CHIKV
488 replication, we took a reverse genetic approach, in which the
489 $_{63}$AUUAAU$_{68}$>$_{63}$CAACUU$_{68}$ mutations were incorporated into both the *trans*-
490 complementation assay and infectious CHIKV. While $_{63}$AUUAAU$_{68}$>$_{63}$CAACUU$_{68}$
491 disrupted the MSI binding site, it was predicted to have no effect on local RNA
492 structure (Supplementary data 4A and B). Similar to previous results following MSI

493 inhibition, expression of both ORF-1 and ORF-2 from the *trans*-complementation
494 system were significantly inhibited in $_{63}$CAACUU$_{68}$-mut relative to the wild type at 8
495 and 24 hpt (Fig 8A and B); indicating that mutation of the putative MSI binding site
496 significantly inhibited CHIKV at the level of virus genome replication. Interestingly,
497 despite repeated attempts, we were not able to rescue infectious CHIKV $_{63}$CAACUU$_{68}$-
498 mut virus, indicating that disruption of the potential MSI binding site completely
499 inhibited infectious CHIKV replication (Fig 8C). In further reverse genetic analysis, we
500 analysed the effect of substitution $_{A}$67$_{G}$ on infectious CHIKV replication. $_{A}$67$_{G}$
501 modified the $_{63}$AUUAAU$_{68}$ to match the published MSI canonical binding site but
502 disrupted adjacent downstream RNA structure (Supplementary data 4C). Following
503 infection at MOI 0.1 in RD cells, CHIKV $_{A}$67$_{G}$ mutant and wild-type control virus was
504 harvested at 8 and 24 hpi and titred by plaque assay (Fig 8D). Compared to wild-
505 type, mutant $_{A}$67$_{G}$ significantly inhibited CHIKV replication at 8 and 24 hpi, indicating
506 that nucleotides $_{63}$AUUAAU$_{68}$ are constrained by both a requirement to interact with
507 MSI-2 and also to maintain essential local RNA structure.
508
509 **Ro 08-2750 further inhibited CHIKV genome replication in $_{63}$CAACUU$_{68}$-mut**
510 In previous experiments, we demonstrated that mutations to disrupt the $_{63}$AUUAAU$_{68}$
511 5'UTR MSI binding site or MSI-2 depletion/inhibition restricted CHIKV genome
512 replication. In order to investigate the potential synergistic effects of these
513 approaches we repeated analysis of $_{63}$CAACUU$_{68}$-mut *trans*-complementation assay
514 replication, in the presence of Ro 08-2750 (Fig 9). Interestingly, in the presence of Ro
515 08-2750, $_{63}$CAACUU$_{68}$-mut replication was inhibited to a significantly greater level
516 than $_{63}$CAACUU$_{68}$-mut in the absence of Ro 08-2750, at both 8 and 24 hpt. These

517 results are consistent with MSI-2 acting through interaction with $_{63}$AUUAAU$_{68}$ in the
518 5'UTR and a further region of the CHIKV genome present in the sub-genomic *trans*
519 complementation reporter (i.e., 5'UTR, intragenic region or 3'UTR).
520
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527 **DISCUSSION**
528
529 For the first time, in this study we demonstrate that cellular RNA binding protein MSI
530 is required for efficient CHIKV replication. Our data demonstrates that MSI-2
531 interacts directly with the 5' end of the CHIKV genome and is consistent with an
532 interaction at position $_{63}$AUUAAU$_{68}$. A single-stranded region located between two
533 conserved stem-loops and immediately upstream of the AUG start codon (Fig 1) (10).
534 Inhibition of MSI-2 expression by siRNA or shRNA, its RNA-binding activity by Ro 08-
535 2750 and reverse genetic disruption of the CHIKV-5'UTR/MSI-2 interaction, inhibited
536 CHIKV replication at the level of virus genome replication. In further analysis, we
537 demonstrated that depletion of both MSI-1 and MSI-2 homologues resulted in a
538 synergistic increase in the level of CHIKV inhibition, supporting the premise that both
539 MSI homologues have a redundant pro-viral effect on CHIKV replication.

540

541 While depletion of MSI-2 by siRNA and shRNA clearly demonstrated significant
542 inhibition of CHIKV replication, measured by infectious virus production, CHIKV-SGR
543 replication or a *trans*-complementation assay, we did not observe complete
544 inhibition of virus replication. Western blot analysis indicated that we did not
545 achieve complete ablation of MSI-2 expression by either siRNA or shRNA,
546 presumably reducing the level of CHIKV inhibition observed. We demonstrated by
547 western blot that in RD cells MSI-2 is expressed to a high level but the MSI-1
548 homologue is only expressed to a low level. However, given the synergistic effect
549 that we observed when silencing expression of both MSI-2 and MSI-1 in Huh7 cells
550 (in which both are expressed to high levels), the ability of MSI homologues to
551 complement for each other may also have reduced the level of CHIKV inhibition
552 observed. This hypothesis is consistent with reverse genetic results, in which
553 $_{63}$AUUAAU$_{68}$> $_{63}$CAACUU$_{68}$ mutation of the MSI binding site, dramatically inhibited
554 CHIKV-5'UTR/MSI-2 binding affinity and completely prevented rescue of infectious
555 mutant virus.

556

557 Mutation $_{63}$AUUAAU$_{68}$> $_{63}$CAACUU$_{68}$ also significantly inhibited replication in the
558 *trans*-complementation assay. However, genome replication was not completely
559 ablated. These differences may indicate that this region has a further function during
560 infectious virus replication, that is not assayed in the *trans*-complementation assay,
561 such as during virion assembly. Further reverse genetic analysis, in which
562 substitution $_{A}$67$_{G}$ engineered a canonical MSI binding site but was predicted to
563 disrupted downstream RNA structure, significantly inhibited infectious CHIKV

564 replication, suggesting that $_{63}$AUUAAU$_{68}$ is constrained by both a requirement to bind
565 MSI and to maintain local RNA structure.
566
567 Translation and replication of positive-sense RNA virus genomes, such as those of
568 CHIKV are mutually exclusive processes – with translation initiating at the 5' end of
569 the genome and replication at the 3'. Consequently, it is essential that such viruses
570 have mechanisms for temporal control of both processes. In many such viruses (e.g.
571 Hepatitis C virus and Polio virus) control involves dynamic interactions between RNA
572 structures in the virus genome and host/virally expressed protein complexes (8). The
573 mechanisms and interactions which CHIKV and other alphaviruses use for temporal
574 control of genome translation and replication remain unclear. However, a recent
575 study suggested a crucial role for the cellular helicase DHX9. Matkovic et al,
576 demonstrated that interaction between DHX9 and the 5' end of the virus genome
577 upregulates CHIKV ORF-1 non-structural protein translation, while simultaneously
578 inhibiting replication of its genome (27). Build-up of nsP2 caused proteosome
579 induced degradation of DHX9, although the detailed mechanism for this remains
580 unclear.
581
582 While, genome replication of positive-sense RNA viruses initiates at the 3' end, it is
583 commonly controlled by promoter elements and interactions with RNA binding
584 proteins at the 5' end. Results described in this and previous studies are consistent
585 with a model in which temporal control of CHIKV ORF-1 translation and genome
586 replication is controlled by a mechanism involving interactions between DHX9, MSI-2
587 and the 5' region of the virus genome. However, the dynamics and mechanism by

588 which the opposing roles if MSI-2 and DHX-9 influence CHIKV replication and their
589 interactions with other regions of the virus genome remains unclear. For example,
590 EMSA and reverse genetic results presented here clearly demonstrate that MSI-2
591 binds to nucleotides $_{63}$AUUAAU$_{68}$ in the 5'UTR and that this interaction is required for
592 efficient CHIKV genome replication. However, the synergistic effect of Ro 08-2750
593 and $_{63}$CAACUU$_{68}$-mut are consistent with MSI-2 also interacting with another region
594 of the CHIKV genome. Bioinformatic analysis has not predicted further MSI binding
595 motifs within the CHIKV genome. Consequently, we hypothesise that any such
596 alternative interactions may be indirect, via a protein complex and are the focus of
597 ongoing studies.
598
599 In summary, using MSI-2 depletion and/or Ro 08-2750 small molecule inhibitor we
600 demonstrate that MSI-2 RNA binding protein is a critical host factor for efficient
601 CHIKV replication but is not required for ORF-1 translation. Inhibition of ORF-1 and
602 ORF-2 signal from the *trans*-complementation assay indicate that MSI-2 is required
603 at the level of CHIKV genome replication. Furthermore, EMSA and reverse genetic
604 results are consistent with a direct interaction between MSI-2 and $_{63}$AUUAAU$_{68}$ in the
605 positive genomic strand of the virus 5'UTR, suggesting a role in negative strand
606 synthesis. Results from this study are important both for our understanding of the
607 fundamental interactions essential to replication of this important human pathogen
608 and for future studies towards specific antiviral therapies.
609

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Data availability statement

The data underlying this article will be shared on reasonable request to the corresponding author.

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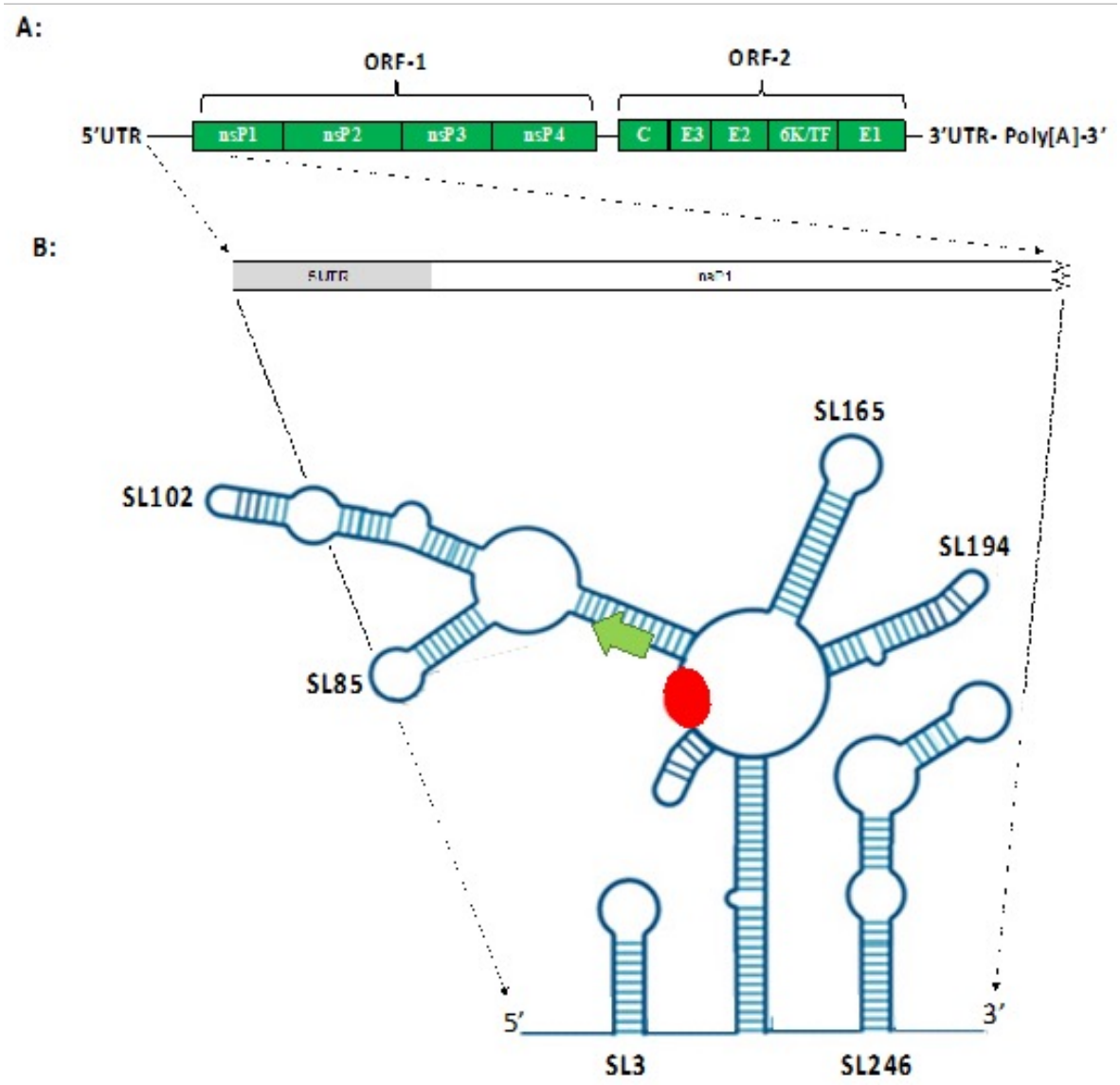
711
712 **Figure Legends:**
713
714 **Figure 1. A)** Schematic representation of CHIKV genome organisation **B)** Schematic
715 representation of CHIKV RNA structures within the 5'UTR and adjacent ORF-1 region
716 of the CHIKV genome (Kendall et al., 2019). RNA replication elements SL3, SL47,
717 SL88, SL102, SL165, SL194 and SL246 are labelled in black type. The ORF-1 AUG start
718 codon is labelled by a green arrow and the putative MSI binding site by a red oval.
719
720 **Figure 2)** Native EMSAs between *in vitro* transcribed P^{32} 5' radiolabeled CHIKV RNA
721 nts 1-330 (*RNA^{WT}) and recombinantly expressed MSI-2 demonstrated an
722 RNA/protein interaction that was outcompeted by increasing concentrations of
723 equivalent unlabeled (RNA^{WT}) but less efficiently by the same RNA incorporating
724 BSM mutation $_{63}AUUAAU_{68}$ >$_{63}CAACUU_{68}$ (RNA^{BSM}). **A)** Increasing concentrations of
725 MSI-2 intensified the observed band shift to the larger RNA/Protein complex and
726 decreased the equivalent unbound RNA band. The interaction between a 1:4 ratio of
727 *RNA^{WT}:MSI-2 was competed with increasing concentrations of unlabelled **B)** RNA^{WT}
728 or **C)** RNA^{mut} ($_{63}CAACUU_{68}$-mut). **D)** Band shifts in the unlabeled RNA competition
729 EMSAs were quantified by densitometry and expressed as % change in the density of
730 the RNA/Protein complex bands, normalized to the equivalent total lane density, for
731 each competition ratio and compared each time to ratio 1:0. N=3, error bars
732 represent standard error from the mean and significance was measured by two-
733 tailed T-test (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). Grey block triangles
734 indicate increasing concentrations of specific reactants.
735

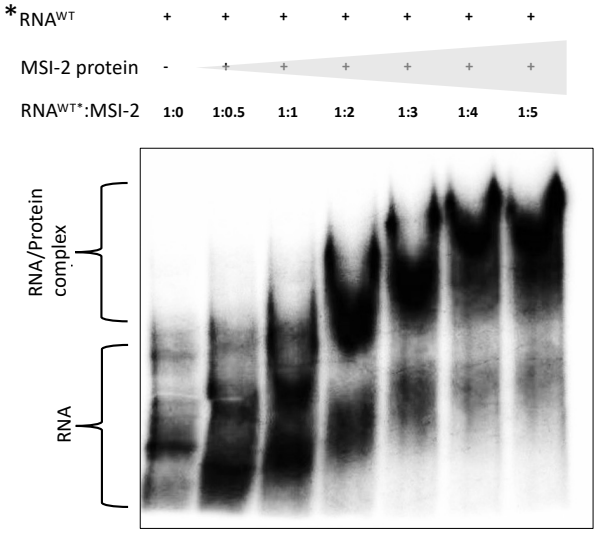
736 **Figure 3.** Ro 08-2750 signifyingly inhibits replication of infectious CHIKV and the
737 CHIKV-SGR **A)** Schematic representations of CHIKV infectious clone (top) compared
738 to the sub-genomic replicon (SGR) (bottom) in which a *Renilla* luciferase (RLuc)
739 reporter gene is fused within the nsp3 coding sequence and the structural genes of
740 ORF-2 are replaced by a firefly luciferase (Fluc) reporter gene. Replication is
741 expressed in Relative Light Units [RLU] **B)** Ro significantly inhibits productive CHIKV
742 productive replication relative to DMSO treated negative controls at 8 and 24 hpi. **C)**
743 and **D**) Ro 08-2750 significantly inhibits CHIKV-SGR replication, measured by both
744 ORF-1 and ORF-2 expression, relative to DMSO treated negative controls at 8 and 24
745 hpt. **E)** Ro 08-2 750 had no significant (ns) effect on CHIKV-SGR (GDD-GAA)
746 translation, measured by ORF-1 expression. N=3, error bars represent standard error
747 from the mean and significance was measured by two-tailed T-test (* = P < 0.05, ** =
748 P < 0.01, *** = P < 0.001).
749
750 **Figure 4.** Ro signifyingly inhibits CHIKV genome replication. **A)** Schematic
751 representation of CHIKV *trans*-complementation assay showing codon optimised
752 pCHIKV-nsP1234 (top) from which the CHIKV nsPs were translated and pCHIK-
753 Fluc/Gluc (bottom) in which ORF-1 was replaced by an Fluc reporter gene, fused to
754 the first 77 nts or CHIKV ORF-1 (N77) downstream of the authentic CHIKV 5'UTR.
755 ORF-2, flanked by the authentic intragenic (SG) and 3' UTRs, was replaced by a Gluc
756 reporter gene. **B)** and **C)** Ro significantly inhibited CHIKV genome replication of the
757 *trans*-complementation assay, measured by both ORF-1 and ORF-2 expression,
758 relative to DMSO treated negative controls at 8 and 24 hpt. N=3, error bars
759 represent standard error from the mean and significance was measured by two-
760 tailed T-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001).
761
762 **Figure 5.** shRNA suppression of MSI-2 significantly inhibits replication of infectious
763 CHIKV. **A)** Western blot analysis of total cellular protein extracted from Rd cells and
764 compared to negative control scrambled shRNA, demonstrated consistent shRNA
765 knockdown of MSI-2 over 3 serial passages (P1- P3). MSI-2 suppression significantly
766 inhibited productive CHIKV replication, relative to scrambled shRNA at 8 and 24 hpi

767 measured by plaque assay **(B)** and strand specific qRT-PCR for the the virus genomic
768 **(C)** and negative intermediate **(D)** strands. N=3, error bars represent standard error
769 from the mean and significance was measured by two-tailed T-test (* = P < 0.05, ** =
770 P < 0.01, *** = P < 0.001).
771
772 **Figure 6.** shRNA suppression of MSI-2 significantly inhibits CHIKV-SGR replication and
773 CHIKV genome replication. **A)** and **B)** shRNA suppression of MSI-2 significantly
774 inhibited CHIKV-SGR replication, measured by both ORF-1 and ORF-2 expression,
775 relative to scrambled shRNA negative controls at 8 and 24 hpt. **C)** and **D)** shRNA
776 suppression of MSI-2 significantly inhibited CHIKV genome replication of the *trans*-
777 complementation assay, measured by both ORF-1 and ORF-2 expression, relative to
778 DMSO treated negative controls at 8 and 24 hpt. N=3, error bars represent standard
779 error from the mean and significance was measured by two-tailed T-test (* = P <
780 0.05, ** = P < 0.01, *** = P < 0.001).
781
782
783 **Figure 7:** siRNA Depletion of either MSI-1 or MSI-2 significantly inhibited CHIKV
784 replication in Huh7 cells and co-depletion of both MSI-1 and MSI-2 had a synergistic
785 effect on CHIKV inhibition in Huh7 cells. siRNA depletion of MSI-2 significantly
786 inhibited CHIKV replication in RD cells and co-depletion of both MSI-1 and MSI-2 did
787 not increase the level of CHIKV inhibition. siRNA depletion of MSI-1 and MSI-2
788 significantly inhibited CHIKV replication in Huh7 cells relative to scrambled siRNA at 8
789 and 24 hpi measured by plaque assay **(A)** and strand specific qRT-PCR for the the
790 virus genomic **(B)** and negative intermediate **(C)** strands. siRNA depletion of MSI-2 in
791 RD cells significantly inhibited CHIKV replication in relative to scrambled siRNA at 8
792 and 24 hpi measured by plaque assay **(D)** and strand specific qRT-PCR for the the
793 virus genomic **(E)** and negative intermediate **(F)** strands. N=3, error bars represent
794 standard error from the mean and significance was measured by two-tailed T-test (*
795 = P < 0.05, ** = P < 0.01, *** = P < 0.001).
796

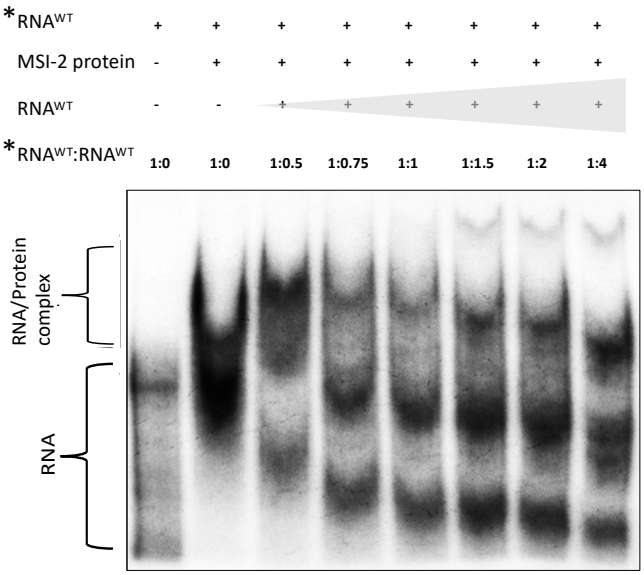
797 **Figure 8.** Substitutions disrupting the predicted MSI binding site ($_{63}$CAACUU$_{68}$-mut)
798 but maintaining local RNA structure prevented virus rescue and significantly
799 inhibited CHIKV genome replication. Substitution $_{A}67_{G}$ maintained the predicted MSI-
800 2 binding motif but disrupted local RNA structure and significantly inhibited CHIKV
801 replication. **A)** and **B)** Mutation $_{63}$CAACUU$_{68}$ significantly inhibited CHIKV genome
802 replication of the *trans*-complementation assay, measured by both ORF-1 and ORF-2
803 expression, relative to wild-type positive controls at 8 and 24 hpt. **C)** Mutation
804 $_{63}$CAACUU$_{68}$-mut prevented rescue of CHIKV following transfection of capped *in vitro*
805 transcribed RNA into BHK cells. Released virus was measured by plaque assay of
806 supernatant 24 hpt and compared to positive control wild-type infectious CHIKV *in*
807 *vitro* transcribed RNA, which was transfected and analysed in parallel. **D)** Mutation
808 $^{A}67^{G}$ significantly inhibited CHIKV replication at 8 hpi and 24 hpi relative to wild type
809 CHIKV following infection of RD cells (MOI 0.1). N=3, error bars represent standard
810 error from the mean and significance was measured by two-tailed T-test (* = P <
811 0.05, ** = P < 0.01, *** = P < 0.001).
812
813 **Figure 9.** Combining substitutions within the predicted MSI binding site
814 ($_{63}$CAACUU$_{68}$-mut) and Ro 08-2750 had a synergistic affect, significantly increasing
815 inhibition of CHIKV genome replication. **A)** and **B)** Mutation of the MSI binding site
816 combined with Ro 08-2750 significantly increased inhibition of the CHIKV *trans*-
817 complementation assay, measured by both ORF-1 and ORF-2 expression, relative to
818 $_{63}$CAACUU$_{68}$-mut in the absence of Ro 08-2750 at 8 and 24 hpt. N=3, error bars
819 represent standard error from the mean and significance was measured by two-
820 tailed T-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001).
821
822


A:
ORF-1
ORF-2
5'UTR
nsP1
nsP2
nsP3
nsP4
C
E3
E2
6K/TF
E1
3'UTR- Poly[A]-3'
B:
SL165
SL102
SL194
SL85
5'
3'
SL3
SL246

A:


*RNA^WT + + + + + + +
MSI-2 protein - + + + + + +
RNA^WT*:MSI-2 1:0 1:0.5 1:1 1:2 1:3 1:4 1:5
RNA/Protein complex
RNA

B:


*RNA^WT + + + + + + + +
MSI-2 protein - + + + + + + +
RNA^WT - - + + + + + +
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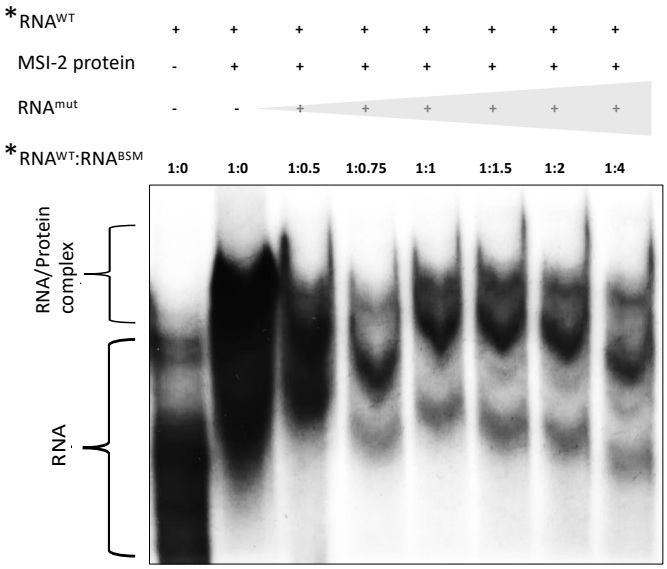
RNA/Protein complex
RNA

C:


*RNA^WT + + + + + + + +
MSI-2 protein - + + + + + + +
RNA^mut - - + + + + + +
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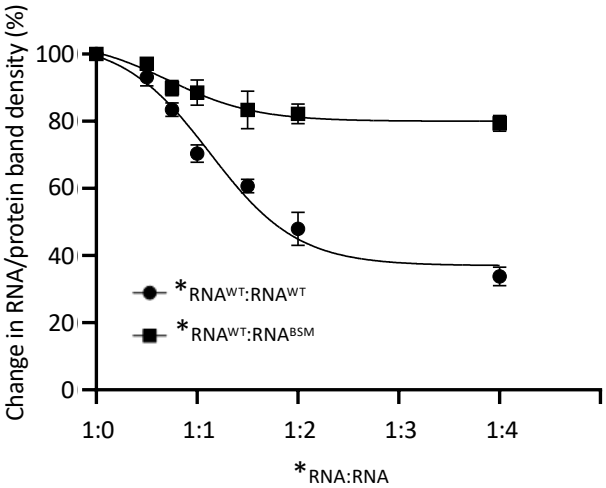
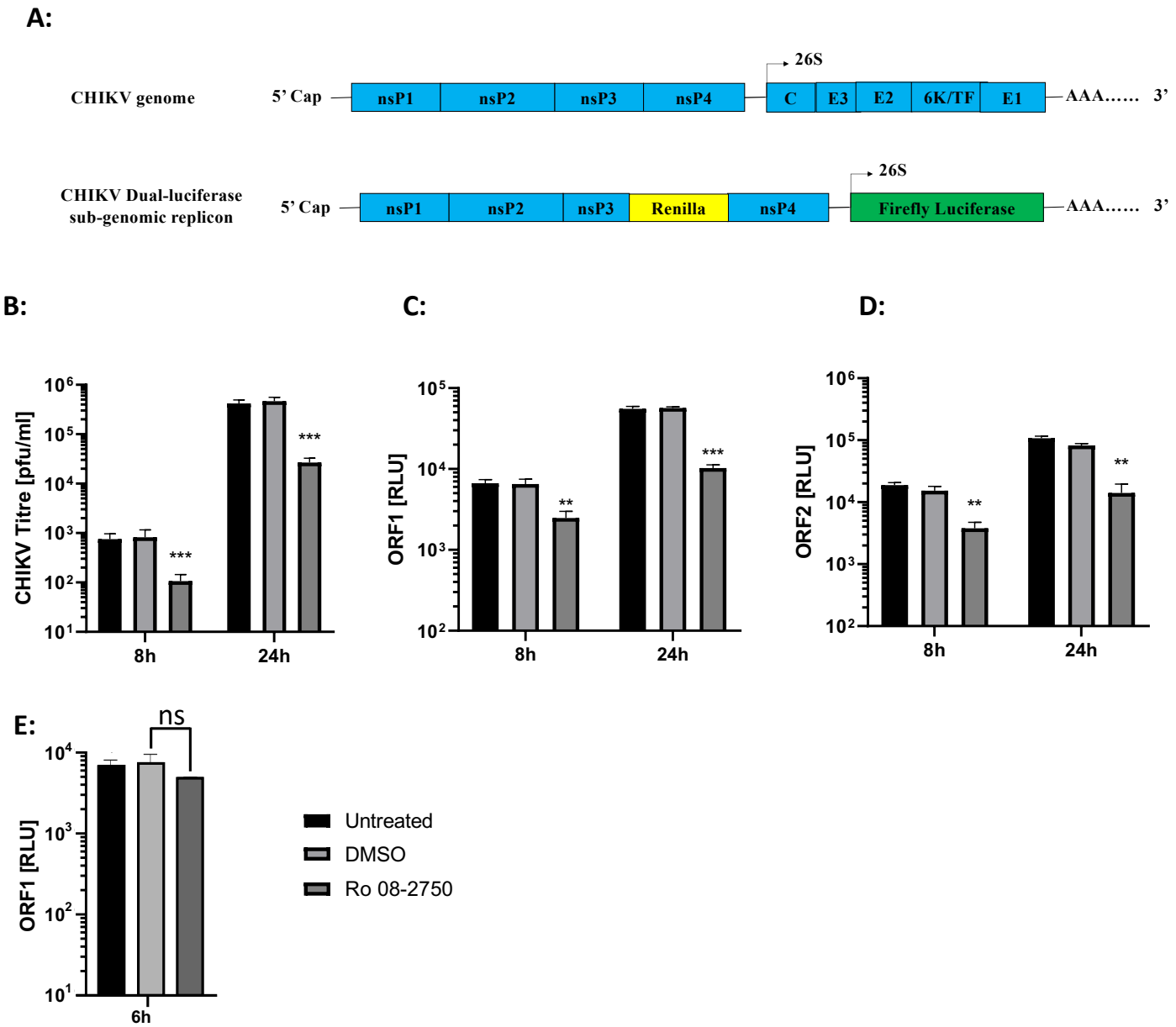
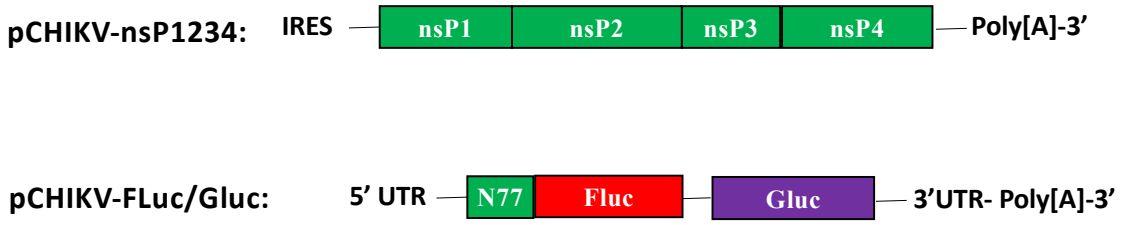
RNA/Protein complex
RNA

D:

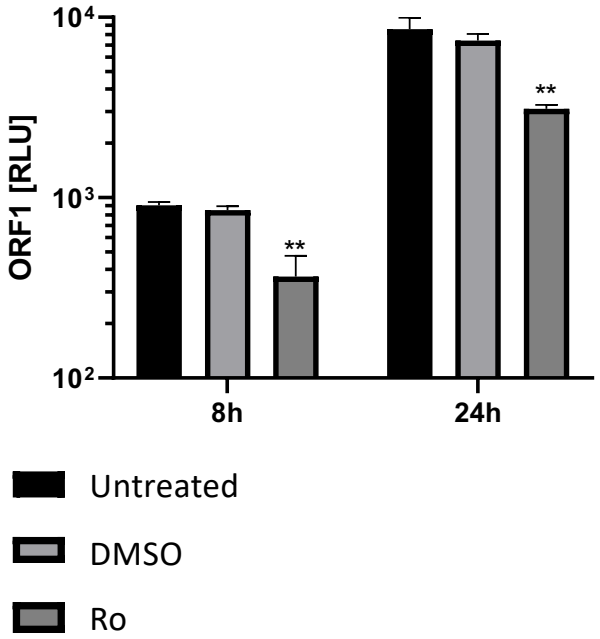

Change in RNA/protein band density (%)
100
80
60
40
20
0
*RNA^WT:RNA^WT
*RNA^WT:RNA^BSM
1:0
1:1
1:2
1:3
1:4
*RNA:RNA


A:
CHIKV genome
5' Cap
nsP1
nsP2
nsP3
nsP4
26S
C
E3
E2
6K/TF
E1
AAA…… 3'
CHIKV Dual-luciferase sub-genomic replicon
5' Cap
nsP1
nsP2
nsP3
Renilla
nsP4
26S
Firefly Luciferase
AAA…… 3'
B:
CHIKV Titre [pfu/ml]
8h
24h
C:
ORF1 [RLU]
8h
24h
D:
ORF2 [RLU]
8h
24h
E:
ORF1 [RLU]
ns
6h
Untreated
DMSO
Ro 08-2750

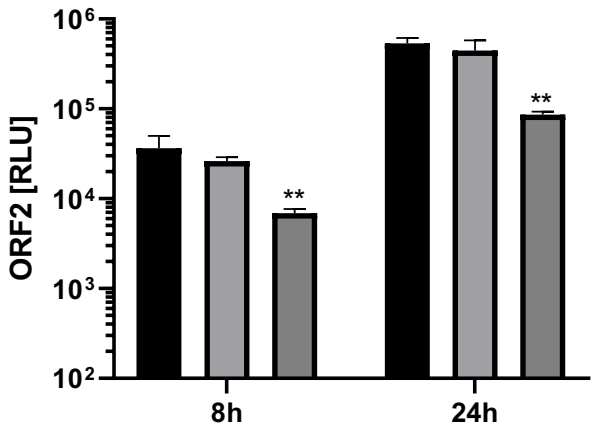
A:

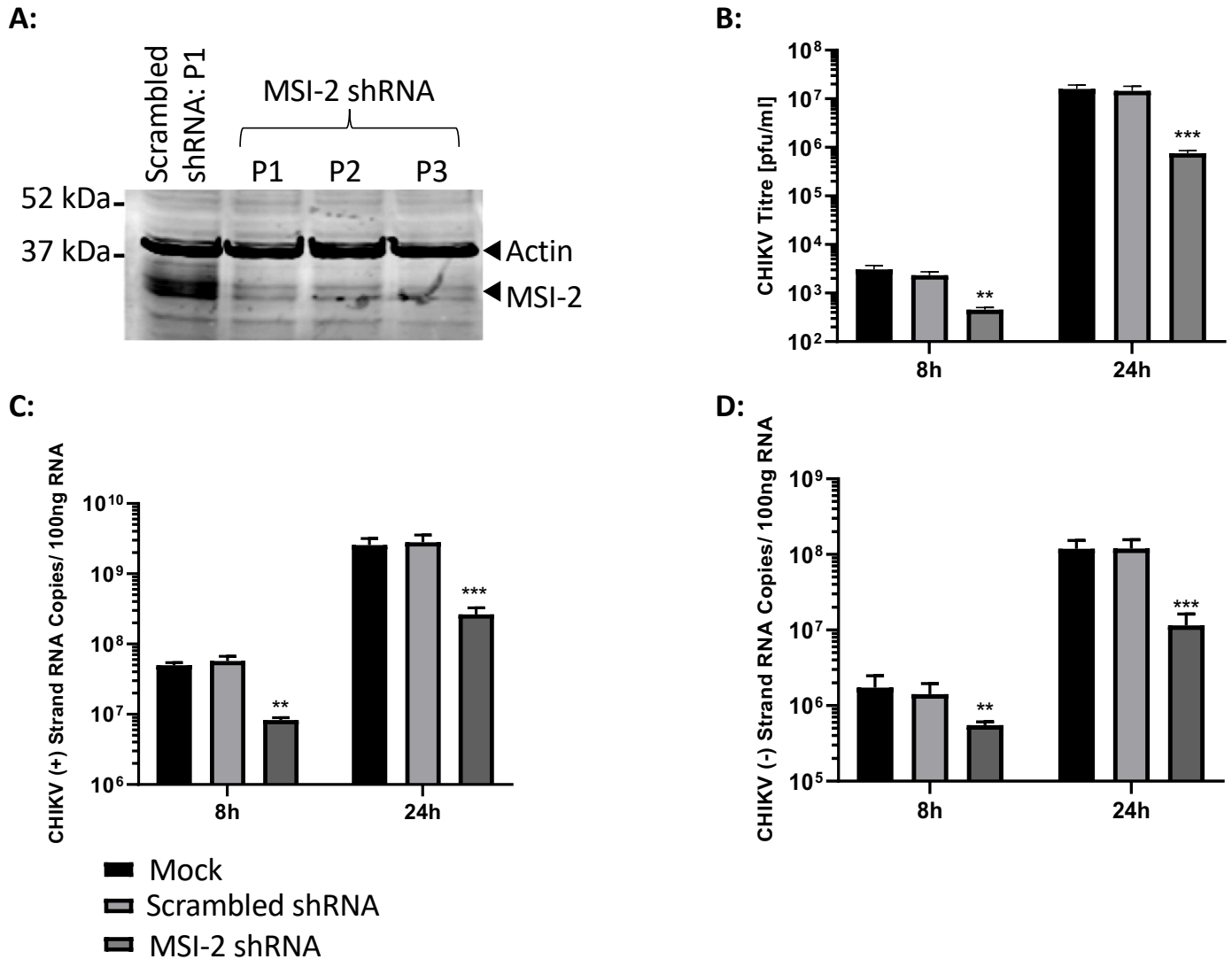

pCHIKV-nsP1234:
IRES
nsP1
nsP2
nsP3
nsP4
Poly[A]-3'
pCHIKV-FLuc/Gluc:
5' UTR
N77
Fluc
Gluc
3'UTR- Poly[A]-3'

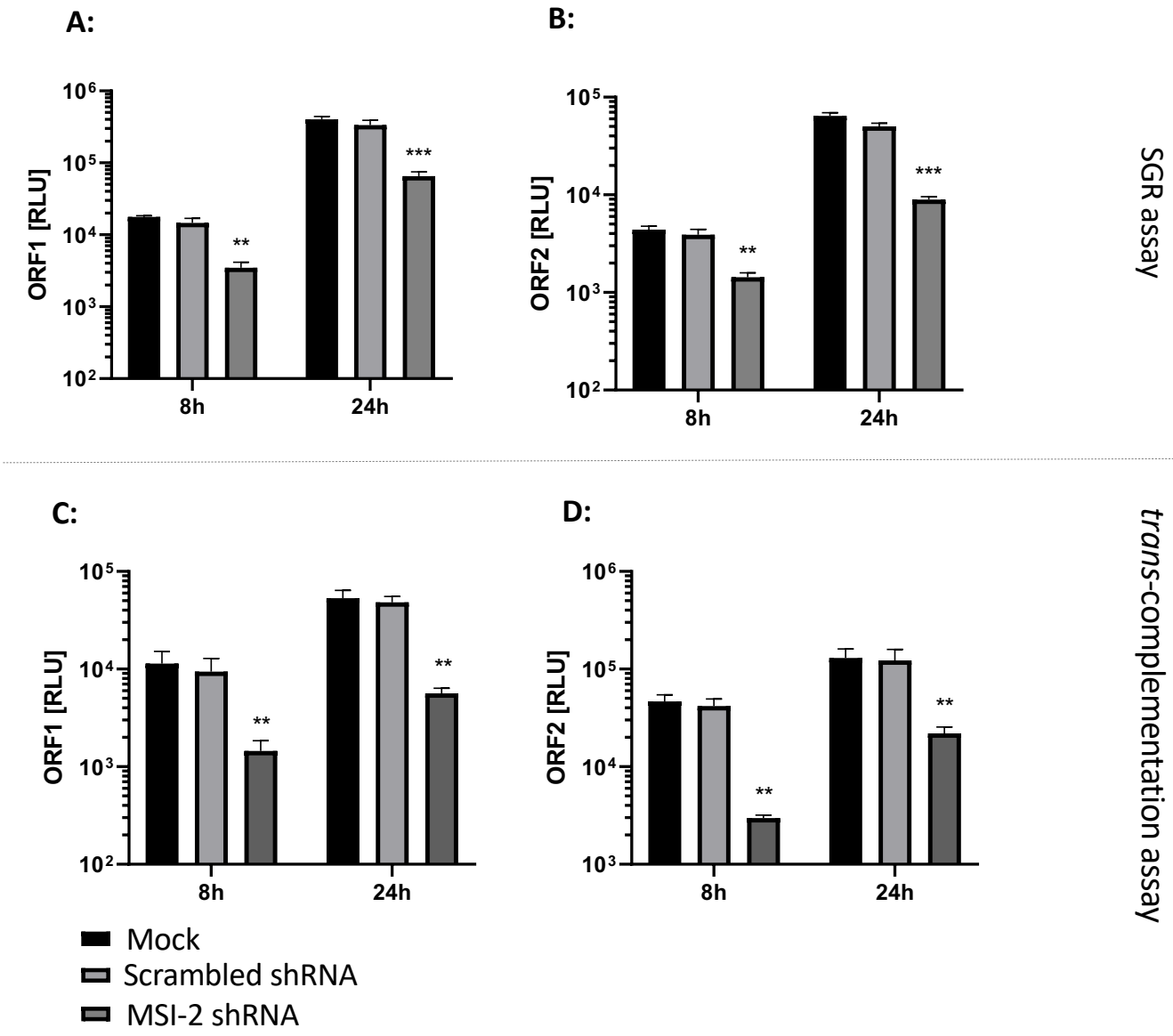
B:

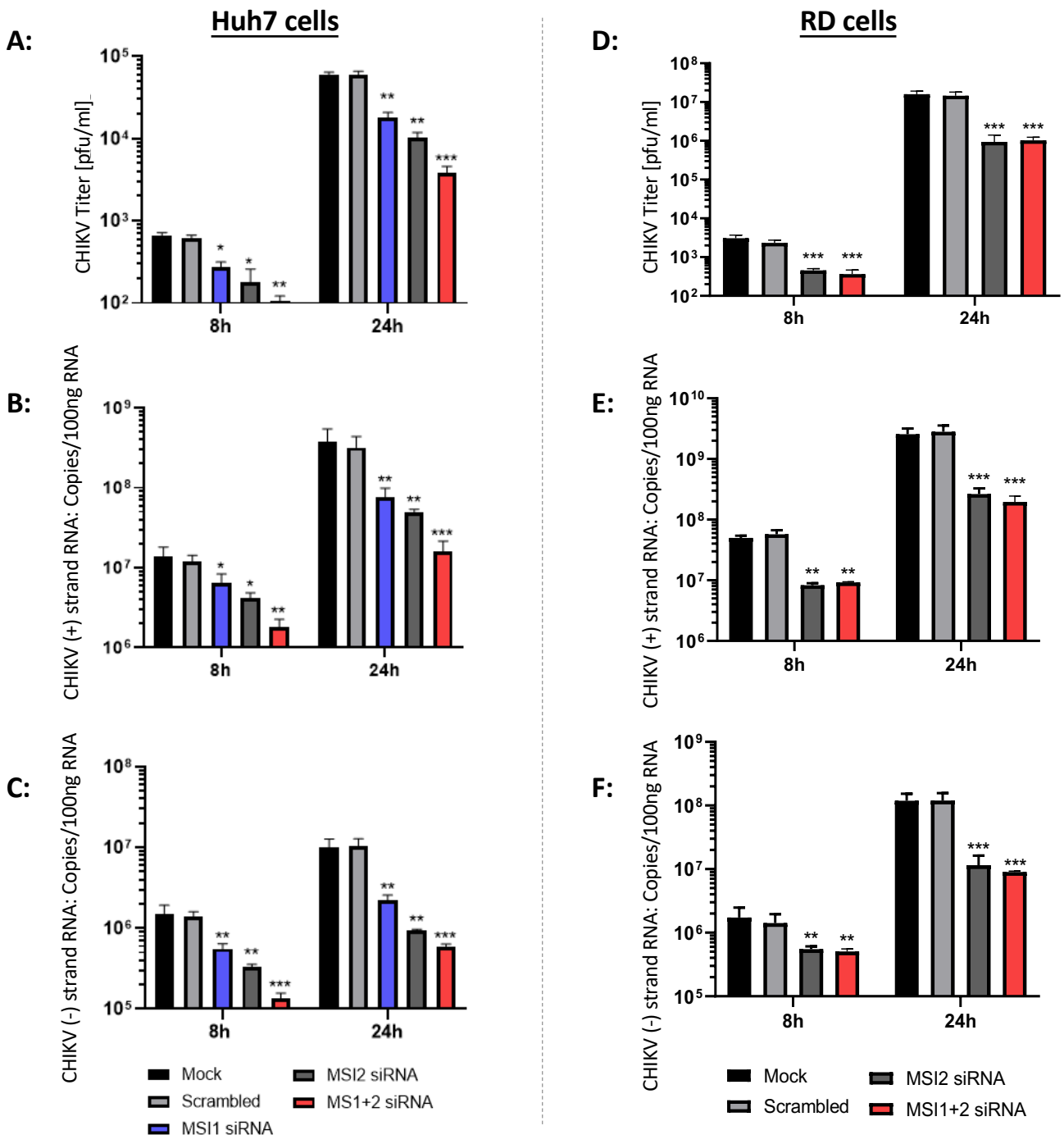

ORF1 [RLU]
10^4
10^3
10^2
**
**
8h
24h
Untreated
DMSO
Ro

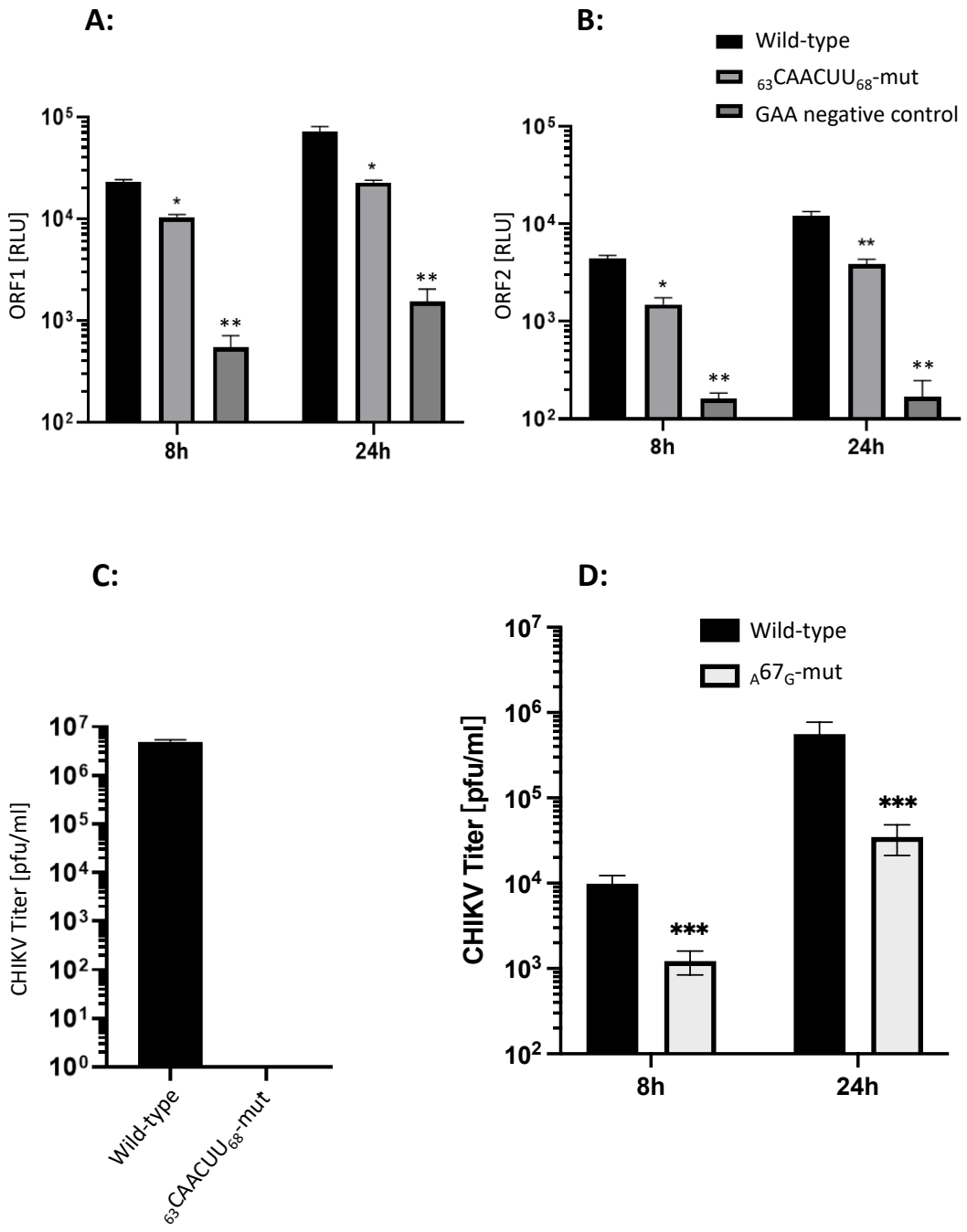
C:


ORF2 [RLU]
10^6
10^5
10^4
10^3
10^2
**
**
8h
24h

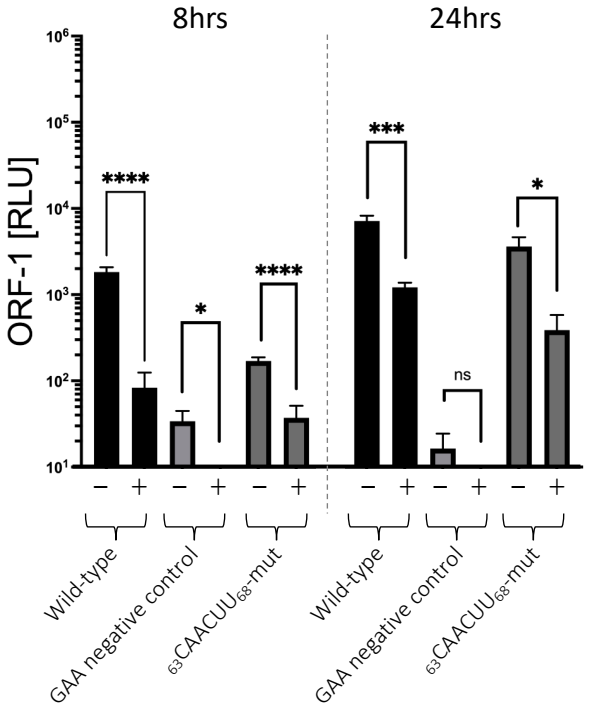

A:
Scrambled shRNA: P1
MSI-2 shRNA
P1
P2
P3
52 kDa
37 kDa
Actin
MSI-2
B:
CHIKV Titre [pfu/ml]
8h
24h
C:
CHIKV (+) Strand RNA Copies/ 100ng RNA
D:
CHIKV (-) Strand RNA Copies/ 100ng RNA
Mock
Scrambled shRNA
MSI-2 shRNA


A:
B:
C:
D:
ORF1 [RLU]
ORF2 [RLU]
8h
24h
**
***
SGR assay
trans-complementation assay
Mock
Scrambled shRNA
MSI-2 shRNA

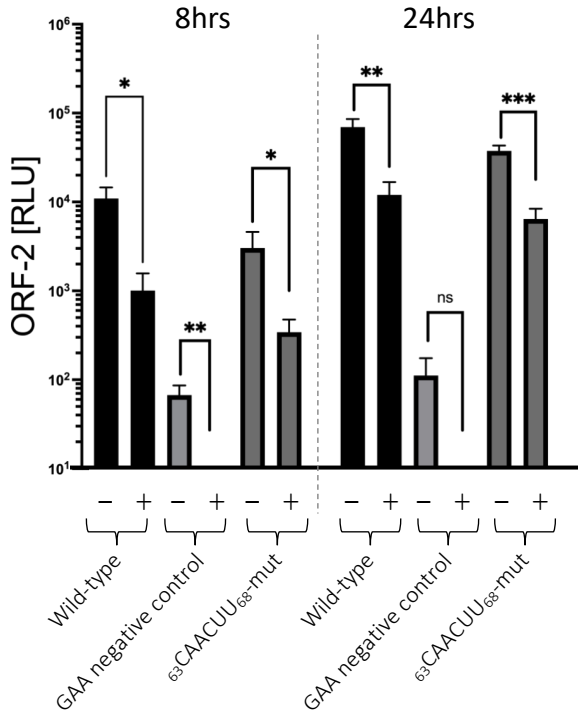

Huh7 cells
RD cells
A:
B:
C:
D:
E:
F:
CHIKV Titer [pfu/ml]
CHIKV (+) strand RNA: Copies/100ng RNA
CHIKV (-) strand RNA: Copies/100ng RNA
8h
24h
Mock
Scrambled
MSI1 siRNA
MSI2 siRNA
MS1+2 siRNA


A:
B:
Wild-type
63CAACUU68-mut
GAA negative control
ORF1 [RLU]
ORF2 [RLU]
8h
24h
C:
D:
CHIKV Titer [pfu/ml]
Wild-type
63CAACUU68-mut
A67G-mut

A:



B:



- = Ro 08-2750 Absent
+ = Ro 08-2750 Present

Supplementary data 1: Alignment of CHIKV 5' region (nts 61-120) showing variation within 5'UTR MSI-2 binding site. All complete CHIKV genomes were downloaded from the NCBI, by using the selection for complete genomes and search term for CHIKV. Duplicates were removed, leaving one representative of each, which were aligned using CLUSTAL OMEGA (multiple sequence alignment) on EBI default settings. MSI-2 binding site indicated by orange box.

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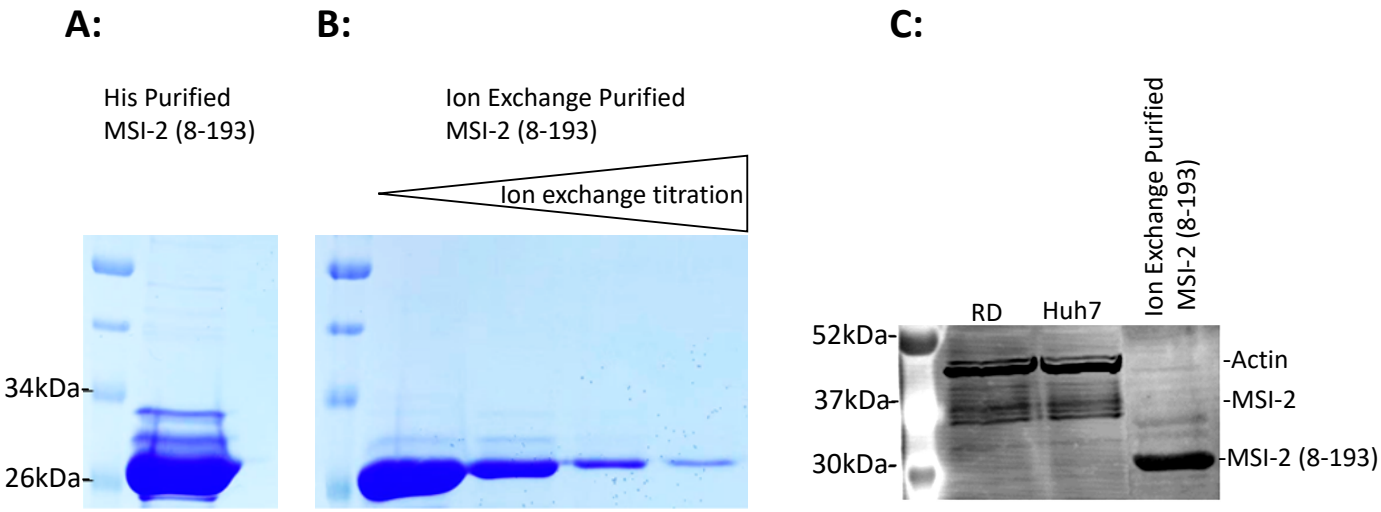
Supplementary data 2: Primers for the reverse transcription and quantitative PCRs for CHIKV strand-specific detection.

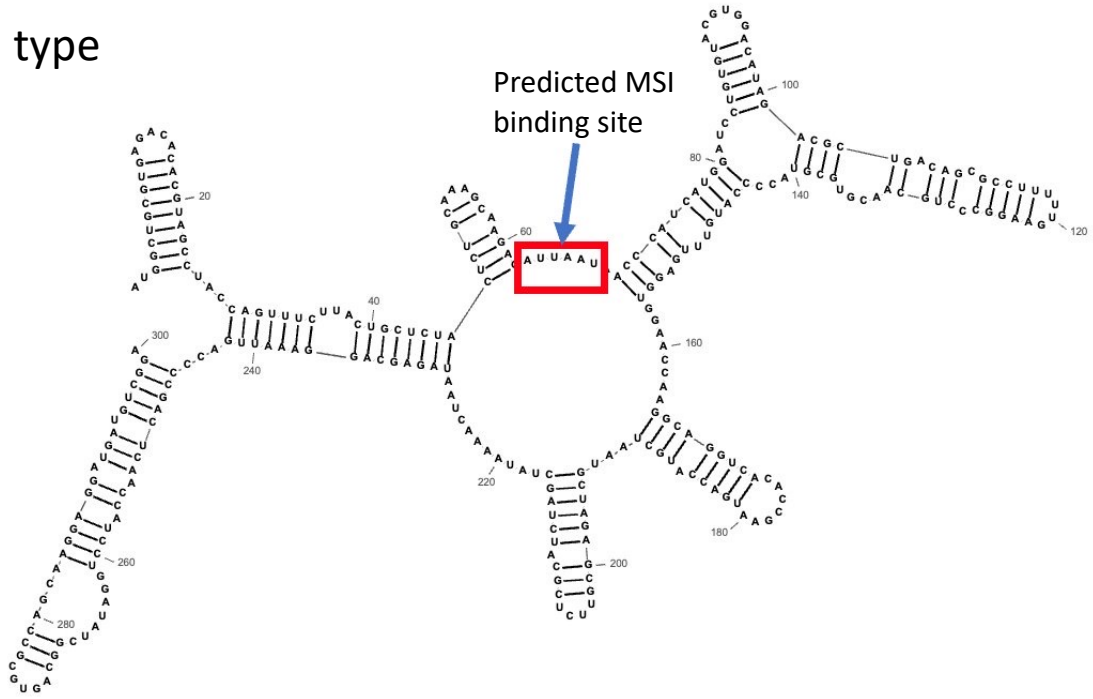
CHIKV (-) strand detection	PCR	Primer sequence (5'-3')
CHIKV FT tag T	reverse transcription	GGC AGT ATC GTG AAT TCG ATG CGA CAC GGA GAC GCC AAC ATT
Tag T	quantitative	GGC AGT ATC GTG AAT TCG ATG C
CHIKV R T	quantitative	AAT AAA TCA TAA GTC TGC TCT CTG TCT ACA TGA
CHIKV (+) strand detection	PCR	Primer sequence (5'-3')
CHIKV RT tag T	reverse transcription	GGC AGT ATC GTG AAT TCG ATG CGT CTG CTC TCT GTC TAC ATG A
CHIKV F T	quantitative	AAT AAA TCA TAA GAC ACG GAG ACG CCA ACA TT
Tag T	quantitative	see above

Supplementary data 3: Expression and purification of MSI-2 Coomassie stained PAGE analysis following **A)** His Tag and **B)** Ion exchange chromatography. **C)** Ion exchange purified MSI-2 (8-193) analyzed by western blot, relative to total protein extracted from RD and Huh7 cells.

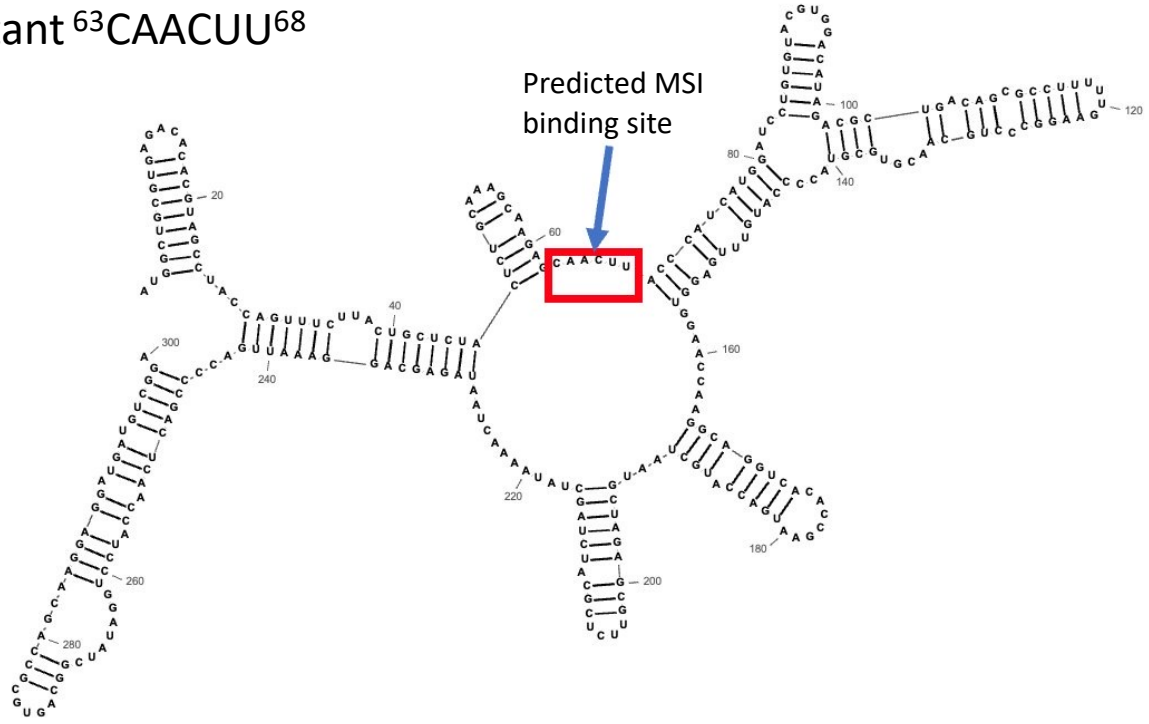


Supplementary data 4: CHIKV predicted RNA structure (nts 1-300) in **A)** wild type **B) m**utant ^{63}CAACUU^{68} and **C)** mutant ^{A}67^{G}. RNA structure mapped free energy minimization using Pfold on default settings.

A) Wild type

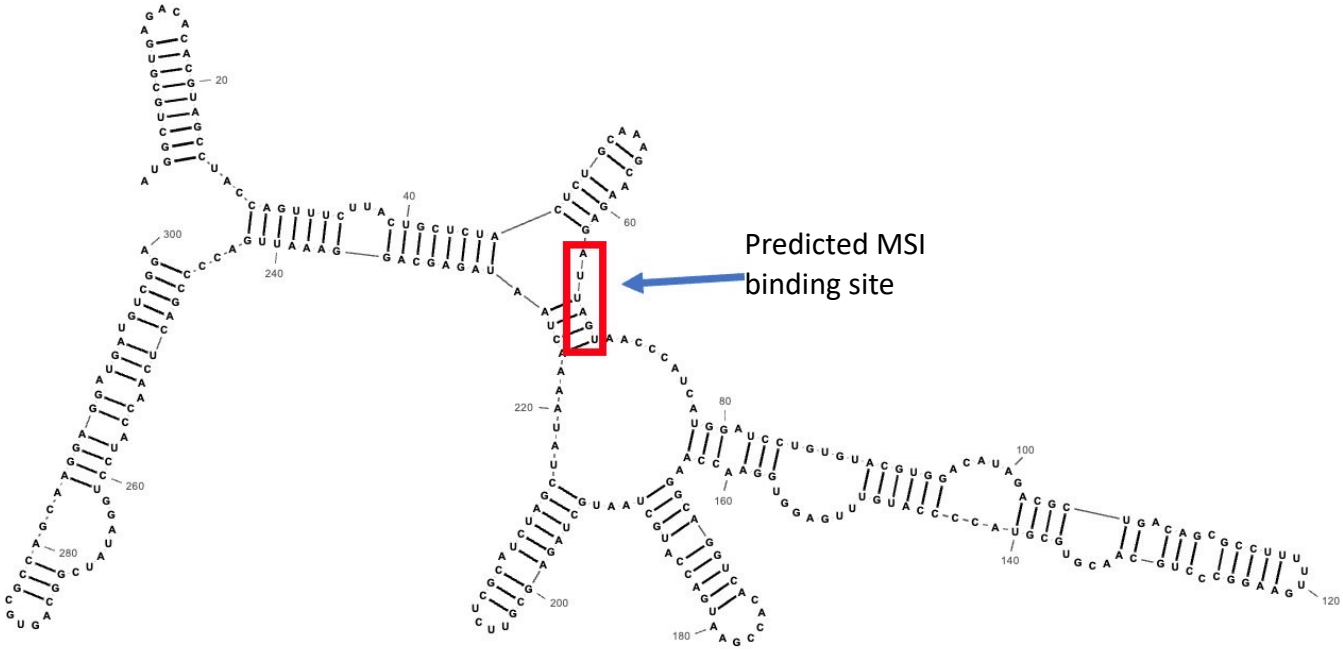


B) Mutant ^{63}CAACUU^{68}

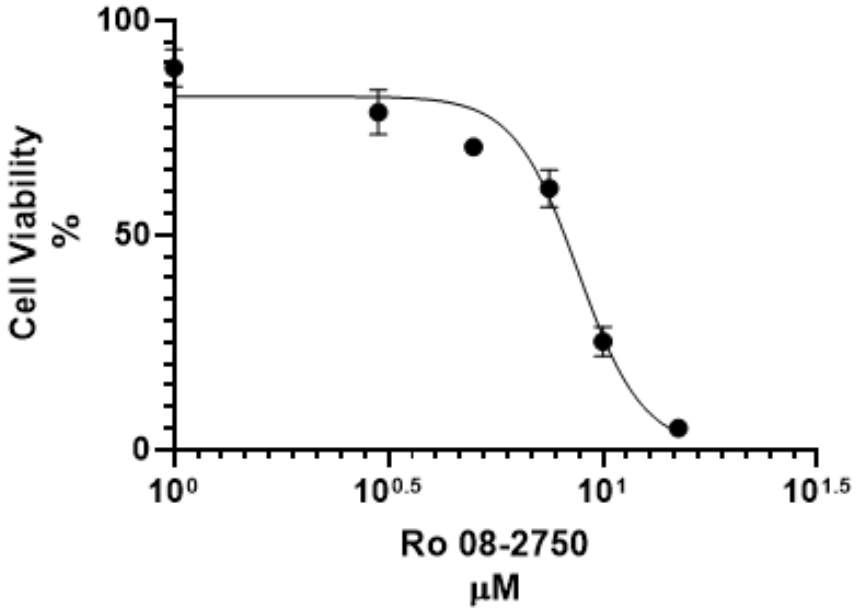


Supplementary data 4: CHIKV predicted RNA structure (nts 1-300) in **A)** wild type **B) m**utant ^{63}CAACUU^{68} and **C)** mutant $^{A}67^{G}$. RNA structure mapped free energy minimization using Pfold on default settings.

C) Mutant $^{A}67^{G}$

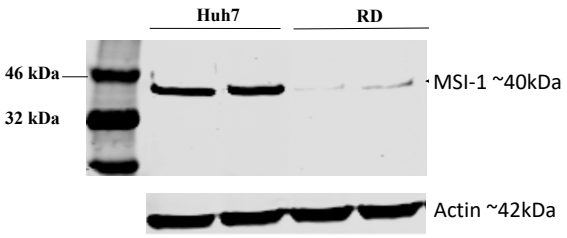


Supplementary data 5: MTT cytotoxicity assay for Ro 08-2750 in RD cells across a titration of 0, 05, 1, 3, 5 10 and 20uM. N=3, error bars represent standard error from the mean.

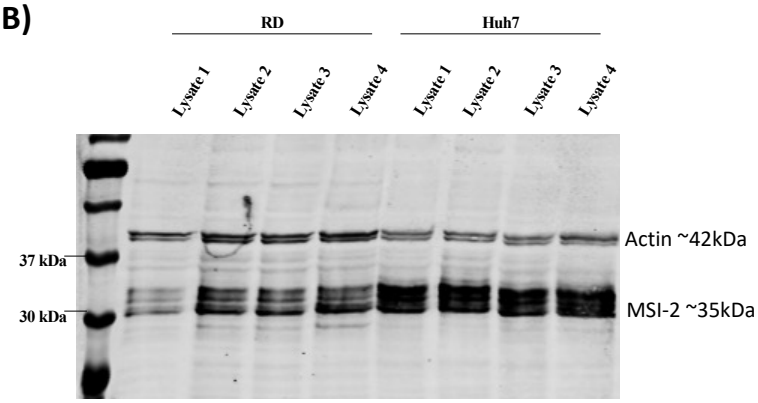
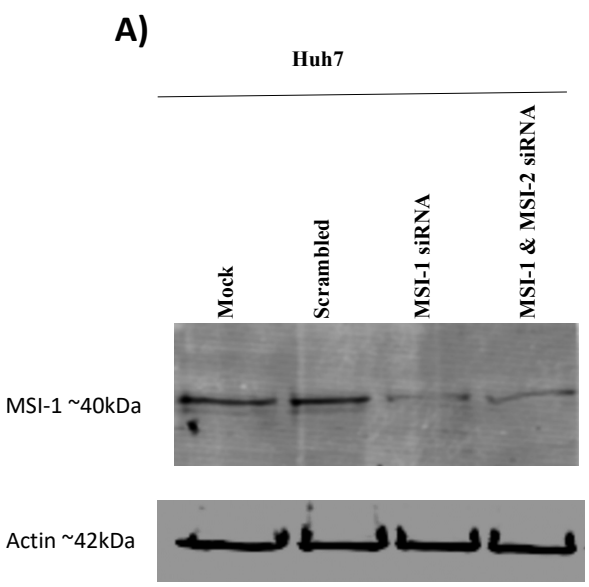
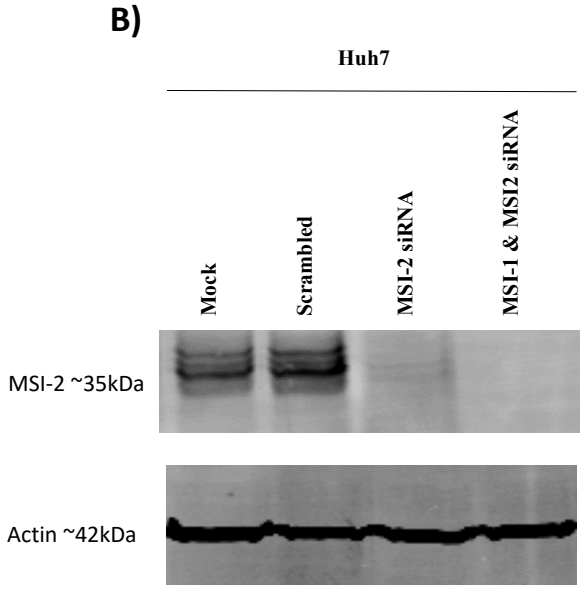
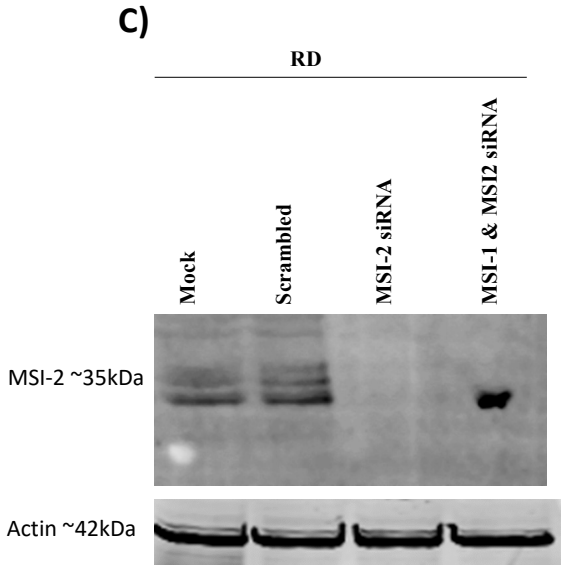


Supplementary data 6: A) MSI-1 and **B**) MSI-2 expression in RD and Huh7 cell lysate analyzed by western blot.

A)



B)



Supplementary data 7: Co-inhibition of MSI-2 and MSI-1 by siRNA in **A)** and **B)** Huh7 cells and **C)** RD cells