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1 Pleotropic effects of mutations in the effector domain of influenza A virus NS1 protein
2
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48
49 ## Abstract

50

51 Objective: The multifunctional NS1 protein of influenza A virus has roles in antagonising
52 cellular innate immune responses and promoting viral gene expression. To better
53 understand the interplay between these functions, we tested the effects of NS1 effector
54 domain mutations known to affect homo-dimerisation or interactions with cellular PI3
55 kinase or Trim25 on NS1 ability to promote nuclear export of viral mRNAs.

56

57 Results: The NS1 dimerisation mutant W187R retained the functions of binding cellular
58 NXF1 as well as stabilising NXF1 interaction with viral segment 7 mRNAs and promoting
59 their nuclear export. Two PI3K-binding mutants, NS1 Y89F and Y89A still bound NXF1 but no
60 longer promoted NXF1 interactions with segment 7 mRNA or its nuclear export. The Trim25-
61 binding mutant NS1 E96A/E97A bound NXF1 and supported NXF1 interactions with segment
62 7 mRNA but no longer supported mRNA nuclear export. Analysis of WT and mutant NS1
63 interaction partners identified hsp70 as specifically binding to NS1 E96A/E97A. Whilst these
64 data suggest the possibility of functional links between NS1's effects on intracellular
65 signalling and its role in viral mRNA nuclear export, they also indicate potential pleiotropic
66 effects of the NS1 mutations; in the case of E96A/E97A possibly via disrupted protein folding
67 leading to chaperone recruitment.

68

69 Keywords: influenza A virus, NS1, PI3K, Trim25, RIG-I, nuclear export

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72 **Introduction**

73

74 Influenza A virus (IAV) infects both man and animals, including species important for food
75 security [1]. A better understanding of its replication cycle is important to aid control
76 measures. Like all viruses, IAV co-opts multiple cellular systems for replication and also has
77 to counteract innate immune defence mechanisms. A key virally-encoded factor in both
78 processes is the NS1 protein. As a nuclear-replicating virus, IAV utilises the NXF1 cellular
79 mRNA export pathway to direct several of its mRNAs to the cytoplasm [2] and we and
80 others have recently found that NS1 acts as an adaptor between segment 7 mRNAs
81 (encoding the M1 or M2 proteins) and cellular mRNA processing/export pathways [3, 4].
82 NS1 is also the primary viral antagonist of innate immune responses, interfering with the
83 function of multiple cellular polypeptides including PKR, 2'-OAS, CPSF30 and Trim25 [5]. NS1
84 also activates the phosphoinositide 3-kinase (PI3K) complex, possibly to inhibit apoptosis
85 [6]. Structurally, NS1 consists of an N-terminal RNA-binding domain and a C-terminal
86 "effector" domain followed by a disordered tail, with no identified enzymatic activities.
87 Instead, it functions by binding to multiple different proteins and RNAs (viral and cellular)
88 and altering their activities [5]. Various NS1 point mutations have been identified that
89 inhibit these interactions and comparative analyses of the behaviour of wild type (WT) and
90 mutant IAV strains with these alterations have provided important information on the
91 biological significance of each particular function. Here, we tested whether mutations
92 identified as affecting NS1 interactions with the p85ß subunit of PI3K (Y89A and Y89F),
93 Trim25 (E96A/E97A) and NS1 effector domain-mediated oligomerisation (W187R) [7-11]
94 also affected NS1's ability to promote nuclear export of segment 7 viral mRNAs.

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96

97 **Methods.**

98 All IAV plasmid constructs and viruses were based on the A/PR/8/34 (PR8) strain described
99 previously [12]. NS1 mutations used in this study were introduced by standard PCR
100 mutagenesis reactions; primer sequences are available on request. Methods for virus
101 reverse genetics, infection, minireplicon assays, fluorescent *in situ* hybridization (FISH),
102 immunofluorescence and confocal microscopy, GFP-trap pulldowns, western blots and
103 reverse transcriptase-radioactive primer extension are described elsewhere [4]. LC-MS
104 analysis was performed using a RSLCnano system (Thermo Fisher Scientific) coupled to a
105 micrOTOF QII mass spectrometer (Bruker) on in-gel trypsin digested protein bands excised
106 from Coomassie Blue-stained SDS-PAGE gels [13]. Raw spectral data were processed to peak
107 lists and searched using Mascot 2.4 server (Matrix Science) against the Uniprot Human
108 sequence database containing 93,786 entries.

109

110 **Results.**

111 Our previous work led to the hypothesis of NS1 acting as an adaptor protein to feed
112 viral segment 7 mRNA(s) into the cellular NXF1 export pathway, by forming a ternary
113 complex between NS1, NXF1 and the mRNA that requires both the NS1 effector domain and
114 a functional RNA-binding domain [4]. To further test the role of the NS1 effector domain, a
115 set of point mutations in this region of the protein were tested (Table 1), firstly in a subviral
116 "minireplicon" system. 293T cells were transfected with plasmids to produce segment 7
117 ribonucleoproteins (RNPs) as well as with either WT or mutant NS1s as EGFP-fusion
118 proteins. Cells were fixed 24 h post transfection and segment 7 mRNA localisation detected

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120 **Table 1. Genetic and phenotypic summary of the NS1 polypeptides used in this study.**

| NS1 protein | mRNA export | NXF1 binding | M1 mRNA binding | Functional alteration | Reference |
|---|---|---|---|---|---|
| WT | + | + | + | n/a | [12] |
| N81[a,b] | - | - | - | Effector domain deletion | [14] |
| R38A/K41A [b] | - | - | - | dsRNA-binding | [15] |
| F103S/M106I [b] | + | + | + | Gain of CPSF inhibition | [16] |
| Y89A | - | + | - | PI3K-binding | [17] |
| Y89F | - | + | - | PI3K-binding | [8] |
| E96A/97A | - | + | + | Trim25-binding | [9] |
| W187R | + | + | + | Homo-dimerisation | [11] |

121 [a]Stop codon added at position 82. [b]See [4] for experimental data.

122

123 by FISH (**Figure 1A**). NS1 expression was confirmed by detection of GFP signal as well as by
124 western blot, although the E96A/E97A mutant accumulated to lower levels than the other
125 NS1s (**Figure 1B**). WT NS1-GFP and most mutant proteins localised largely to the cytoplasm,
126 but the W187R mutant was found both in nucleus and cytoplasm (**Figure 1A**). No mRNA
127 staining was detected in the negative control lacking a complete RNP. As expected [4],
128 when the minimal components for RNP reconstitution were present, segment 7 mRNA was
129 detected primarily in cell nuclei, whilst on addition of NS1-GFP, it translocated to the
130 cytoplasm. However, in the presence of PI3K-binding mutants NS1-Y89A or NS1-Y89F or the
131 Trim25-binding mutant NS1-E96A/E97A, segment 7 mRNA was clearly still retained in the
132 nucleus. In contrast, the dimerization mutant NS1-W187R caused segment 7 mRNA to
133 localise to the cytoplasm. To quantify these effects, cells were visually scored according to
134 the predominant cellular localisation of segment 7 mRNA (nuclear, cytoplasmic or both). In
135 the absence of NS1, segment 7 was mainly nuclear in ~80% of cells scored, while the
136 remaining cells presented mRNA staining both in the nucleus and the cytoplasm (**Figure 1C**).
137 When WT NS1 or NS1-W187R were added, a statistically significant shift of segment 7 mRNA
138 localisation was confirmed, with ~ 75% of cells showing mRNA in the cytoplasm. However,

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139 addition of NS1-E96A/E97A, NS1-Y89A or NS1-Y89F did not produce this shift, with a clear
140 majority of cells displaying nuclear segment 7 mRNA, similarly to samples with no NS1-GFP.
141 To further test the mRNA export activity of the NS1 effector domain mutants, their
142 phenotype was examined in the context of virus infection. 293T cells were mock infected or
143 infected with either WT PR8 or the NS1 mutant viruses and at 6 h post infection (p.i.), fixed
144 and processed for segment 7 FISH analysis as well as immunofluorescently stained for NS1
145 (**Figure 1D**). All viruses generally gave cytoplasmic NS1 signal above the uninfected cell
146 background, although staining of cells infected with NS1-Y89 and NS1-E96A/E97A mutants
147 was noticeably fainter than the WT and W187R viruses. No viral mRNA signal was detected
148 in the mock sample while segment 7 mRNA was cytoplasmic in WT and W187R virus-
149 infected cells. In cells infected with NS1-Y89A, NS1-Y89F and NS1-E96A/E97A, segment 7
150 mRNA was predominantly nuclear. Thus, in both minireplicon and viral settings, the NS1
151 dimerization mutant W187R supported normal segment 7 mRNA nuclear export, but the
152 PI3K-binding and Trim25-binding mutants showed loss-of-function.
153 NS1 both binds NXF1 and promotes the ability of NXF1 to bind segment 7 mRNA [4,
154 18-20]. To test whether the effector domain mutants retained NXF1-binding activity, 293T
155 cells were transfected with either GFP or GFP-NXF1 and 48 h later mock infected or infected
156 with the panel of viruses. At 6 h p.i., cells were lysed and GFP-Trap precipitations were
157 performed on the supernatants. Western blot analyses of total and bound fractions showed
158 that GFP and GFP-NXF1 were both expressed and collected in comparable amounts (**Figure
159 2A**). Blotting for NS1 confirmed successful infection, although the NS1-E96A/E97A
160 polypeptide again accumulated to lower levels than the other NS1s. Nevertheless, all NS1
161 polypeptides co-precipitated with GFP-NXF, indicating that the mutations did not abrogate
162 the interaction.

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163 To test whether the effector domain mutations affected the ability of NS1 to
164 promote a stable interaction between NXF1 and segment 7 mRNA, 293T cells were
165 transfected with GFP or GFP-NXF1 and infected with WT or mutant viruses as above. At 6 h
166 p.i., cells were lysed and total RNA was extracted before or after the lysates had been
167 subjected to GFP-Trap pulldown. Segment 7 RNA species were then detected by primer
168 extension. Viral mRNAs and genomic vRNA were detected in the total fraction of every
169 infected sample (**Figure 2B**, lanes 1-2 and 4-7). Analysis of the bound fraction from WT virus
170 infected cells showed that both segment 7 mRNA species but not genomic vRNA co-
171 precipitated with GFP-NXF1 (lane 9) while no viral RNAs bound to GFP alone (lane 8),
172 consistent with previous work [4, 18]. In contrast, viral mRNAs were not detected in GFP-
173 NXF1 pulldowns from cells infected with either NS1-Y89A or NS1-Y89F viruses (lanes 11 and
174 12). The NS1-W187R and -E96A/E97A mutants also supported NXF1 interactions with the
175 viral mRNAs (lane 13, 14). Since the E96A/E97A mutation blocked successful nuclear export
176 of segment 7 mRNA, this finding indicated that, like the interaction between NS1 and NXF1,
177 the interaction between NXF1 and segment 7 is necessary, but not sufficient for the
178 successful nuclear export of segment 7 mRNA.
179 The behaviour of the NS1 E96A/E97A mutant – unable to mediate nuclear export of
180 segment 7 mRNA despite binding to NXF1 and promoting its interaction with the mRNA –
181 suggested the involvement of another essential factor in the hypothesised ternary export
182 complex. Accordingly, we examined cellular interaction partners of WT NS1 and the
183 mutants, including a complete deletion of the effector domain (NS1-N81), an RNA-binding
184 domain mutant (R38A/K41A) and a gain-of-function mutant that restores CPSF30-binding
185 activity (F103S/M106I), of which only the latter supported mRNA nuclear export (Table 1;
186 [4]). 293T cells were transfected with GFP or GFP-tagged NS1 mutant plasmids. Two days

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187 later, cells were collected, lysed and subjected to GFP-Trap. Bound fractions were loaded
188 onto SDS-PAGE gels and stained with Coomassie Blue. No obvious contamination from
189 cellular GFP-binding proteins was seen from cells transfected with the GFP control plasmid
190 (**Figure 2C**, lane 1). Major polypeptide species of the expected size were detected in all
191 other samples, including the truncated form NS1-N81, confirming successful expression
192 (lanes 2-9). Various other minor polypeptide species were also visible that were mostly
193 common to all NS1-containing samples. However, one clear difference was observed; a
194 distinct novel polypeptide in the E96A/E97A preparation (lane 8) that was absent from
195 other samples. LC-MS analysis of the excised band unambiguously identified this
196 polypeptide as heat shock protein 70 (hsp70) through multiple peptide matches (**Table S1**).
197 To confirm the interaction between NS1-E96A/E97A and hsp70, repeat GFP-trap
198 pulldowns were analysed by western blotting for hsp70 as well as for the GFP-tagged "bait"
199 proteins. Transfection was confirmed by the detection of GFP and GFP-tagged NS1 mutant
200 proteins, while equivalent amounts of the cellular proteins hsp70, Nup62 and UAP56 in the
201 total fractions of all samples confirmed that comparable amounts of cell lysates had been
202 generated (**Figure 2D**, lanes 1-9). However, in the bound fraction, hsp70 was detected only
203 in the NS1-E96A/E97A sample (lane 17), confirming the specific interaction with hsp70 for
204 NS1 E96A/E97A.
205
206 **Discussion**
207 The data reported here extend our previous investigation [4] into the role of NS1 in
208 facilitating nuclear export of viral mRNA. Firstly, we show that higher order multimerization
209 of NS1 via its effector domain is not needed for viral mRNA nuclear export. Secondly, the
210 finding that point mutations in the NS1 effector domain, and not just its complete deletion

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211 along with the C-terminal unstructured region [4], can block viral mRNA nuclear export,
212 strengthens the argument for the functional involvement of this domain in the process.
213 Thirdly, identification of an NS1 mutant (E96A/E97A) that still binds NXF1 and enhances its
214 interaction with segment 7 mRNA without upregulating mRNA nuclear export suggests that
215 at least one other factor is needed for export. Finally, the effect of mutations previously
216 considered to specifically inhibit PI3K activation or inhibition of RIG-I signalling on M1 mRNA
217 localisation could suggest the involvement of one or both of these pathways in viral mRNA
218 nuclear export.

219 Of broader significance than the mechanism of IAV mRNA nuclear export, our data
220 add to the evidence suggesting pleiotropic effects from NS1 mutations commonly used as
221 tools to interrogate viral interactions with the PI3K and RIG-I pathways (*e.g.* [10, 17, 21-
222 28]). Potential pleiotropic effects of the E96A/E97A mutation have been noted previously,
223 including poor expression of the polypeptide as seen here [25] and an intriguing effect on
224 PI3K activation [29]. Indeed a recent report detailing the crystal structure of NS1-Trim25
225 complexes concluded that NS1 residues E96 and 97 do not directly contact Trim25 but
226 instead support a network of contacts within the local region of the effector domain in
227 which L95 and S99 actually make contact with Trim25 [30]. Our finding that the E96A/E97A
228 mutation also induces binding of hsp70 further suggests effects on protein folding and that
229 interpreting results obtained with this mutation may be complicated.

230

231 **Limitations**

232 Attempts to probe the role of PI3K activation in IAV mRNA export using small molecule
233 inhibitors gave inconsistent results (data not shown) and could not be further pursued
234 because of time and funding constraints. It would also have been interesting to test the

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235 effects of hsp70 inhibitors [31] had time allowed. The effect of NS1 mutations L95A/S99A on
236 mRNA export should also be tested.
237

238 ## Declarations

239 **Ethics approval and consent to participate:** not applicable.
240 **Availability of data and material:** available from the corresponding author on reasonable
241 request.
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250 **Consent for publication:** not applicable.
251 **Competing interests:** none to declare.
252 **Author's contributions:** CFP carried out the majority of the experimental work with input
253 from MJA, HW and RMP. DK and ACG performed the LC-MS analyses. CFP and PD drafted
254 the manuscript and all authors read, corrected and approved the final manuscript.
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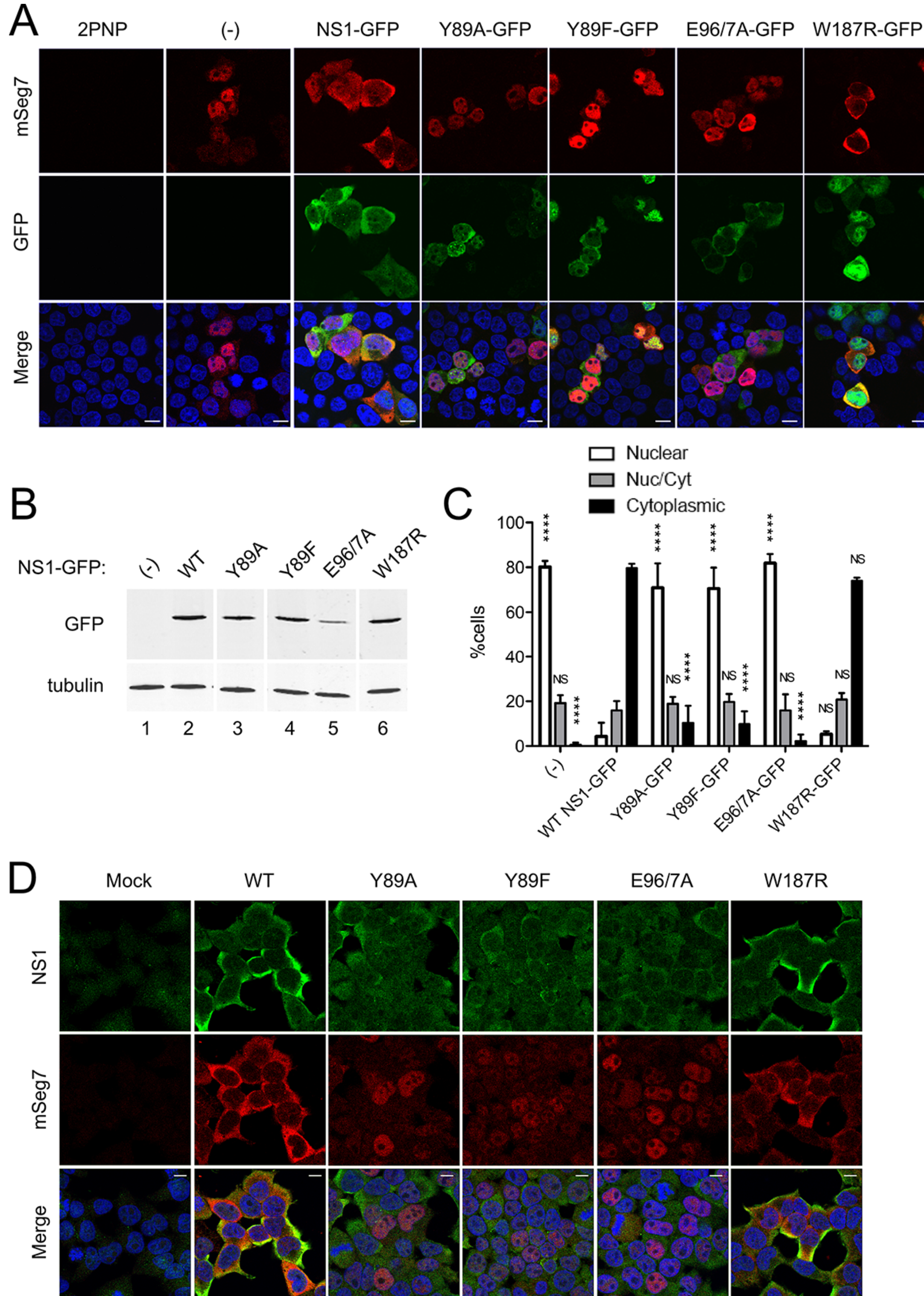
260 ## Figures and Legends

261




262

263

264 **Figure 1. Effect of mutations in the NS1 effector domain on segment 7 mRNA localisation.**

265 A-C) 293T cells were transfected with plasmids to recreate segment 7 RNPs or, as a negative

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266 control with a combination that excluded a plasmid expressing PB2 (2PNP), together with
267 the indicated NS1-GFP polypeptides. (A) Cells were processed for FISH analysis at 24 h post
268 transfection (p.t.) using Cy3-labelled RNA probes for detection of positive sense segment 7
269 viral RNAs (red). GFP fluorescence was also detected (green). Images were captured using a
270 Leica-TCS confocal microscope and Leica TCS analysis software. (B) Duplicate cell samples
271 were lysed and examined by SDS-PAGE and western blotting for GFP and tubulin. (C)
272 Individual cells were scored according to the predominant cellular localisation of segment 7
273 mRNA considering three phenotypes: nuclear, cytoplasmic or mixed. Values are the mean ±
274 range from two independent experiments. A 2-way ANOVA was used to test for statistically
275 significant differences between the values for each category from those obtained for WT-
276 NS1 (NS, not significant; **** $P < 0.0001$). (D) 293T cells were mock infected or infected
277 with the indicated viruses at an M.O.I. of 5. At 6 h post infection (p.i.), cells were processed
278 for FISH as above (red) and stained with anti-NS1 serum (green) and DAPI (for DNA; blue).
279 Scale-bars indicate 10 µm. Lanes 1 and 2 in panel (B) and a portion of the corresponding
280 numerical data in panel (C) are taken with permission from Figure 4C and 4B respectively of
281 [4].
282

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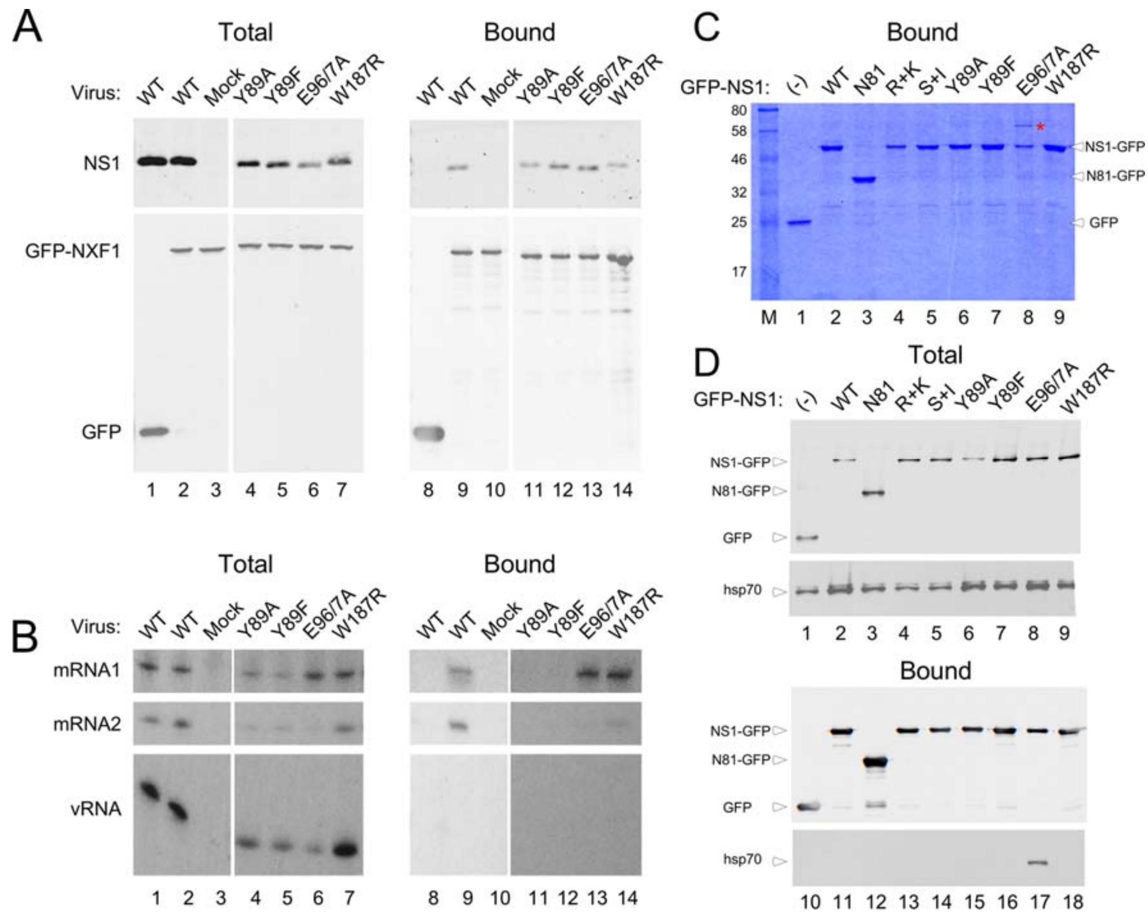


283
284
285 **Figure 2. Interactions between NS1, cellular proteins and segment 7 mRNAs.** (A) 293T cells
286 were transfected with plasmids encoding GFP or GFP-NXF1 and 48 h later either mock
287 infected or infected with the indicated NS1 mutant viruses at an M.O.I. of 5. Cells were
288 harvested at 6 h p.i. and cell lysates examined by western blotting for the indicated proteins
289 before (Total) or after (Bound) being subjected to GFP-Trap pulldown assays. (B) 293T cells
290 were transfected with GFP-NXF1 (GFP-NXF1 +) or with GFP alone (GFP-NXF1 -) and 48 h later
291 either mock infected or infected with the indicated viruses at an M.O.I. of 10, in duplicate
292 sets. At 6 h p.i., cells were harvested and total cellular RNA was extracted from one set
293 while the second set was subjected to GFP-Trap pulldown assays prior to total RNA
294 extraction. RNA was analysed by reverse transcription with radiolabelled primers specific for
295 segment 7 RNAs followed by urea-PAGE and autoradiography. (C, D) 293T cells were
296 transfected with either GFP alone or with GFP-tagged NS1 mutant protein expressing
297 plasmids as indicated and 48 h later cells were harvested and subjected to GFP-Trap pull
298 down assays. Eluted samples were loaded onto SDS-PAGE gels and (C) stained with
299 Coomassie Blue dye or (D) western blotted for GFP or hsp70 before (Totals) or after (Bound)
300 GFP-trap fractionation. Lanes 1-3 and 8-10 from panel (A) are reproduced with permission
301 from Figure 7 of [4].
302

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