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A Single Amino Acid Substitution in the Novel H7N9 Influenza A Virus NS1 Protein Increases CPSF30 Binding and Virulence

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ABSTRACT

Although an effective interferon antagonist in human and avian cells, the novel H7N9 influenza virus NS1 protein is defective at inhibiting CPSF30. An I106M substitution in H7N9 NS1 can restore CPSF30 binding together with the ability to block host gene expression. Furthermore, a recombinant virus expressing H7N9 NS1-I106M replicates to higher titers *in vivo*, and is subtly more virulent, than parental. Natural polymorphisms in H7N9 NS1 that enhance CPSF30 binding may be cause for concern.

Since early 2013, zoonotic transmission of a novel avian-origin H7N9 influenza A virus in eastern China has led to at least 441 human infections and 122 deaths (1). Sequence analyses and functional studies have identified several mammalian-adaptive polymorphisms in PB2 (20, 33), PA (32), HA and NA (7, 13), that may be responsible for promoting replication and pathogenicity of this virus in humans. Nevertheless, it is clear that avian-origin H7N9 has yet to adapt to humans: HA retains its preference for avian receptors (25, 26, 30, 31), and H7N9 is unable to transmit efficiently between humans. Identifying sequence changes in H7N9 that may evolve naturally and contribute to future human-to-human transmission, or which might alter H7N9 virulence, is critical.

Novel H7N9 virus NS1 is an interferon (IFN)-antagonist in human and chicken cells. NS1 is a multifunctional virulence factor that plays a major role in antagonizing host IFN responses during infection [reviewed in (10)]. Notably, this property can vary in efficiency between virus isolates and between host-cell species' origin (11, 12, 15, 17, 24). We tested the ability of H7N9 NS1 to limit production of IFNβ in human and avian cells, and compared it with a panel of NS1 proteins from seasonal human influenza viruses or other avian H5N1 viruses that have sporadically infected humans since 1997 (**Figure 1**). Human 293T, or chicken DF-1, cells were transfected with an IFNβ promoter-driven firefly (FF)-luciferase reporter construct together with expression plasmids for the NS1 proteins of interest (or GST as a control). For analysis of novel H7N9, we tested NS1 proteins derived from two different human H7N9 strains (A/Shanghai/1/2013 [Sh/1] and A/Shanghai/2/2013 [Sh/2]), along with the NS1 from the closely-related avian H9N2 virus, A/Chicken/Dawang/1/2011 [Dw/11]. Subsequent

infection with a DI-rich SeV preparation induced robust amounts of IFNβ promoter-driven FF-luciferase activity in GST-expressing human 293T cells, but not in cells expressing any of the NS1 proteins (**Figure 1A**). Similar results were observed in chicken DF-1 cells, albeit the inhibitory impact of all NS1s in this system was less evident (**Figure 1B**), possibly due to the absence of chicken RIG-I (2), a key target for NS1 in humans (19). Notably, Tx/91 NS1, a well characterized and potent inhibitor of general host gene expression (15), appeared to act as the strongest IFN-antagonist. These data suggest that the human H7N9 and avian H9N2 precursor NS1s can function as IFN-antagonists in human and chicken cells, a finding in agreement with recent results from others (14).

A single I106M substitution in novel H7N9 NS1 enhances CPSF30 binding and inhibition of host gene expression. Binding of NS1 to cellular CPSF30 contributes to the global post-transcriptional inhibition of cellular pre-mRNA processing during infection, and is an additional mechanism by which most human influenza viruses attenuate host antiviral responses (4, 21, 22). This property of NS1 is not conserved in all strains (15, 29), and variation in CPSF30 binding has been reported to arise when viruses are adapted to replicate in certain new host species (5, 23). There are two notable examples of naturally-occurring influenza viruses that have infected humans but which encode NS1 proteins unable to inhibit CPSF30: highly-pathogenic avian-origin 1997 H5N1 virus (29) and the swine-origin 2009 pandemic H1N1 virus (11). The defect in CPSF30 binding maps to slightly different amino-acid positions in the 1997 H5N1 and 2009 pdmH1N1 NS1 proteins (summarized in **Figure 2A**), and opposite phenotypes have

been identified for 'gain-of-function' substitutions that restore CPSF30 inhibition in these two viruses: for 1997 H5N1, gain of CPSF30 binding promotes systemic spread of the virus and increases virulence (27), while for 2009 pdmH1N1, gain of CPSF30 binding slightly decreases replication and virulence (11). Such phenotypic differences may be due to the disparate amino-acid substitutions required to restore binding, or the distinct genetic constellations of the two avian- or swine- origin viruses. Certainly, the impact on replication and virulence of modulating CPSF30 binding affinity is complex, and seemingly unpredictable between strains (3, 5, 11, 23, 27, 28).

Intriguingly, we observed that the novel H7N9 NS1 has the same amino-acid polymorphisms that weaken CPSF30 binding as the 1997 H5N1 NS1 (**Figure 2A**). We tested the co-precipitation of FLAG-tagged CPSF30 with bacterially-expressed 6His-tagged wild-type Sh/2 NS1 (WT), as well as with L103F, I106M or L103F/I106M double mutant (DM) variants that we predicted might have a different CPSF30-binding profile. Only a small amount of FLAG-CPSF30 could be precipitated by NS1-WT or NS1-L103F (**Figure 2B**). However, FLAG-CPSF30 bound efficiently (and equally) to both NS1-I106M and NS1-DM, suggesting that the H7N9 NS1:CPSF30 interaction is indeed weak, but can be strengthened by the single I106M substitution.

To test the ability of H7N9 NS1 to block general host gene expression, we co-transfected 293T cells with a constitutively-active *Renilla*-luciferase reporter construct (pRL-SV40) together with each of the indicated Sh/1 NS1 constructs, and measured total *Renilla*-luciferase activity 24 h later. As shown in **Figure 2C**, the WT H5N1 VN/04 NS1 (that binds CPSF30 (29)) efficiently inhibited *Renilla*-luciferase activity, while the H7N9 NS1-

WT protein (like GST) was deficient in this function. In agreement with our pull-down studies, NS1-L103F was also unable to inhibit *Renilla*-luciferase activity, while both NS1-I106M and NS1-DM proteins efficiently limited activity (**Figure 2C**). These results indicate that the single I106M substitution in H7N9 NS1 restores both CPSF30 binding and the inhibition of cellular gene expression. The I106M substitution specifically enhanced CPSF30 binding, as no differences were observed between NS1-WT and any of the NS1 mutants with regards interaction with RIG-I (19) or TRIM25 (6) (**Figures 2D** & **2E**). The I106M substitution also did not impact upon the co-precipitation of Riplet with NS1 (24), although there was a possible enhancement of this interaction when NS1 constructs contained the L103F substitution (**Figure 2F**). These data highlight a key difference with the 1997 H5N1 NS1 protein, where a similar substitution at position 106 was reported to differentially affect CPSF30 and RIG-I binding (3).

Characterization of an H7N9-based virus expressing NS1-I106M *in vitro*. Using an H7N9 reverse genetics system (9), we generated recombinant WT and NS1-I106M viruses containing the 6 'internal' gene segments of Sh/1 and the HA and NA gene segments from PR8 (H1N1). This 6+2 strategy using the surface antigens of the laboratory PR8 H1N1 strain was adopted for risk mitigation purposes given the 'gain-of-function' nature of the experiment. The two viruses (rSh/1 (6+2) WT and rSh/1 (6+2) NS1-I106M) were plaque-purified, and stocks grown and titrated in MDCKs. The NS genomic segment of each virus from stock aliquots was sequenced to ensure the absence of additional mutations. Surprisingly, multi-cycle replication experiments revealed that the recombinant WT and NS1-I106M viruses grew with identical kinetics to each other in primary differentiated human tracheobronchial epithelial cells (HTBEs; Clonetics, Lonza,

126 Walkersville, MD, USA) (**Figure 3A**). We also assessed ability of the two viruses to
127 repress IFNβ production using an MDCK cell-line stably expressing firefly luciferase
128 (FF-Luc) under control of the IFNβ promoter (8). Notably, both WT and NS1-I106M
129 virus infection led to the same low level (~3-5-fold) induction of the reporter (**Figure**
130 **3B**). The similarity of replication kinetics and IFNβ induction for the two viruses in
131 primary HTBEs was independently confirmed by qRT-PCR (**Figures 3C** & **3D**). These
132 data suggest that, in the context of rSh/1 (6+2), the defect in efficient CPSF30 binding by
133 H7N9 NS1 does not significantly affect virus replication in primary human cells or virus-
134 mediated IFNβ-antagonism *in vitro*. This may be due to the impact of other IFN-
135 antagonists encoded by the virus [reviewed in (18)], or the observation that H7N9 NS1 is
136 an efficient IFN-antagonist (14) and retains affinity for RIG-I/TRIM25/Riplet. It is also
137 possible that partial compensation of the H7N9 NS1:CPSF30 deficiency occurs by the
138 cognate H7N9 viral polymerase complex, as has been shown for 1997 H5N1 (16, 29).
139
140 **An H7N9-based virus expressing NS1-I106M shows enhanced replication and**
141 **virulence *in vivo*.** To test whether increased affinity of H7N9 NS1 towards CPSF30
142 would affect viral replication and pathogenicity *in vivo*, we determined MLD_{50} values for
143 the WT and NS1-I106M viruses in 6-8 week old C57BL/6 mice (Jackson Laboratory,
144 ME). All procedures were performed in accordance with the IACUC guidelines of Icahn
145 School of Medicine at Mount Sinai, and animals showing >25% weight loss were
146 considered to have reached experimental endpoint and were humanely euthanized.
147 MLD_{50} values were subsequently calculated according to the method of Reed and
148 Muench (see **Table 1** for a summary of all the data). Even in the highly-virulent rSh/1

(6+2) background, the NS1-I106M mutation lead to a modest ~2.5-fold increase in MLD_{50}, and mice infected with the NS1-I106M virus exhibited greater overall mortality (**Figure 3E**) and morbidity (as determined by duration and extent of weight loss, and day of death; **Table 1**) than mice infected with WT virus. We speculate that the virulence-enhancing impact of H7N9 NS1-I106M may be even more pronounced in the context of non-PR8 glycoproteins, where the respective WT virus would have a much higher MLD_{50}.

To assess replication *in vivo*, mice were intranasally infected with 500 PFU of each virus and lungs excised on days 2 and 4 post-infection. Following homogenization and centrifugation (10,000 x g, 5 min, 4°C), the resulting supernatants were used to determine viral titer. The NS1-I106M virus replicated to titers >5-fold higher than WT by day 2 (p = 0.0056), while titers at day 4 were similar (**Figure 3F**). Notably, qRT-PCR analysis of lung homogenates from independently infected mice suggested a trend for the NS1-I106M virus to induce less IFNβ mRNA than the WT virus, however this was not statistically significant (**Figure 3G**). These data indicate that, in contrast to *in vitro*, 'gain-of-function' substitutions in NS1 that enhance CPSF30 binding and inhibition of general gene expression slightly enhance the replication and pathogenicity of H7N9-based viruses *in vivo*.

Concluding remarks. Continued zoonotic transmission of H7N9 to humans is a significant cause for concern given the mild to lethal human respiratory disease it causes, and the fear that it may yet acquire human-to-human transmission. Here, we characterize

the H7N9 NS1 protein as an efficient IFN-antagonist. Nevertheless, H7N9 NS1 is defective in binding CPSF30, and is consequently unable to block host-cell gene expression. We identify the single I106M natural polymorphism found in non-H7N9 strains as a potential 'gain-of-function' mechanism by which the H7N9 NS1 could acquire CPSF30 binding, and provide evidence that this substitution promotes virus replication and virulence *in vivo*. These results parallel those found with the 1997 H5N1 virus and the laboratory strain, PR8, where similar substitutions enhanced CPSF30 binding and virulence (27-29). Although polymorphisms in H7N9 NS1 that might restore CPSF30 binding have yet to be identified in the sequenced strains available, our study highlights the importance of continued surveillance to monitor potential natural 'gain-of-function' mutations in H7N9 NS1 that may impact pathogenicity.

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196 **FIGURE LEGENDS**

197

198 **Figure 1. The H7N9 NS1 protein is an IFN-antagonist in human and chicken cells.**
199 (**A**) Human 293T, or (**B**) chicken DF-1, cells were co-transfected for 16 h with a
200 pCAGGS expression plasmid encoding the indicated NS1 protein (or GST) together with
201 a FF-luciferase (FF-Luc) IFNβ-promoter reporter plasmid (p125Luc). After infection
202 with a DI-rich SeV preparation for a further 12 h, FF-Luc activity was determined.
203 Results represent the means and standard deviations of triplicate values (normalized to
204 GST + SeV) obtained in a single experiment, and are representative of two independent
205 experiments. The NS1 sequences (containing silent splice acceptor mutations to prevent
206 NEP/NS2 expression (11)) were derived from: A/Texas/36/91 (Tx/91; human seasonal-
207 like H1N1), A/Wyoming/03/03 (Wy/03; human seasonal-like H3N2), A/California/04/09
208 (Cal/09; human seasonal-like H1N1, previously 2009 pdmH1N1), A/Hong Kong/156/97
209 (HK/97; representative of the 1997 H5N1 outbreak), A/Vietnam/1203/04 (VN/04;
210 representative of the 2004 H5N1 outbreak), A/Shanghai/1/2013 (Sh/1; human H7N9),
211 A/Shanghai/2/2013 (Sh/2; human H7N9) and A/Chicken/Dawang/1/2011 (Dw/11; avian
212 H9N2 virus with NS1 closely-related to the H7N9 NS1).

213

214 **Figure 2. A single I106M substitution in the H7N9 NS1 protein specifically restores**
215 **efficient CPSF30 binding and inhibition of host-cell gene expression.** (**A**) **Summary**
216 **data for a selection of NS1s used in this study.** Asterisk denotes predicted binding
217 affinity based on related H3N2 viruses. **(B) Binding of NS1 to CPSF30.** 293T cell-lysate
218 overexpressing FLAG-CPSF30 was mixed with the indicated bacterially-expressed 6His-

NS1 protein (WT, L103F, I106M, L103F/I106M [DM]; or 6His-MCS negative control) and pulled-down using Ni-NTA beads. Precipitates eluted after extensive washing were analysed by SDS-PAGE and western blotting using anti-NS1 and anti-FLAG antibodies. (**C**) **NS1-mediated inhibition of general host gene expression.** Human 293T cells were co-transfected with a pCAGGS expression plasmid encoding the indicated NS1 protein (or GST) together with a constitutively active *Renilla*-luciferase plasmid. Luciferase activity was determined 24 h post-transfection. Results show the means and standard deviations of triplicate values normalized to GST. Statistical significance (**) was determined using the Student's *t*-test. (**D-F**) **Binding of NS1 to cellular factors involved in the IFN-induction cascade.** Experiments were performed as for (B), using 293T lysates overexpressing FLAG-RIG-I (**D**), V5-TRIM25 (**E**), or HA-Riplet (**F**). Western blotting was performed using appropriate anti-tag antibodies.

Figure 3. Characterization of H7N9-based NS1-WT and NS1-I106M viruses *in vitro* and *in vivo*. (**A**) **Virus replication *in vitro*.** Multicycle growth analysis of rSh/1 (6+2) WT and rSh/1 (6+2) NS1-I106M viruses in primary differentiated human airway epithelial cells (HTBEs). Data points show mean values and error bars represent standard deviation. (**B**) **Induction of IFNβ by the recombinant viruses.** MDCK-IFNβ-FFluc cells were infected at an MOI of 2 PFU/cell for 16 h with the indicated virus (or mock) prior to analysis of luciferase activity. Bars represent mean values (n=3) and error bars represent SD. (**C** & **D**) **qRT-PCR analyses of viral replication and IFNβ induction *in vitro*.** Primary HTBEs were infected at an MOI of 2 PFU/cell and lysed at the times indicated. Total RNA was extracted, and following reverse transcription using oligo(dT),

the levels of viral M1 mRNA (**C**) and IFNβ mRNA (**D**) were quantified in triplicate by qPCR. Values were averaged and normalized to actin mRNA. Mean induction levels relative to mRNA levels in mock-infected cells are shown. Error bars represent standard deviations. Means and standard deviations were calculated from biological triplicates. (**E**) **Virulence in mice.** Survival data for six-to-eight week-old C57/BL6 mice infected intranasally with 10 PFU of the indicated virus (20 mice per virus). Body weights were determined daily for 14 days and mice showing more than 25% weight loss were considered to have reached the experimental endpoint and were humanely euthanized. (**F**) **Virus replication *in vivo*.** Six-to-eight week-old C57/BL6 mice were infected intranasally with 500 PFU of the indicated virus. Lung titers were determined on days 2 and 4 post-infection from 3-4 mice per group. Bars represent mean values. Statistical significance was determined using the Student's *t*-test. (**G**) **qRT-PCR analyses of IFNβ induction *in vivo*.** Six-to-eight week-old C57/BL6 mice (3-4 mice per group) were infected intranasally with 500 PFU of the indicated virus for 1 or 3 days. Murine IFNβ mRNA was quantified from lung homogenates by qRT-PCR as described in panel D.

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Figure 1.

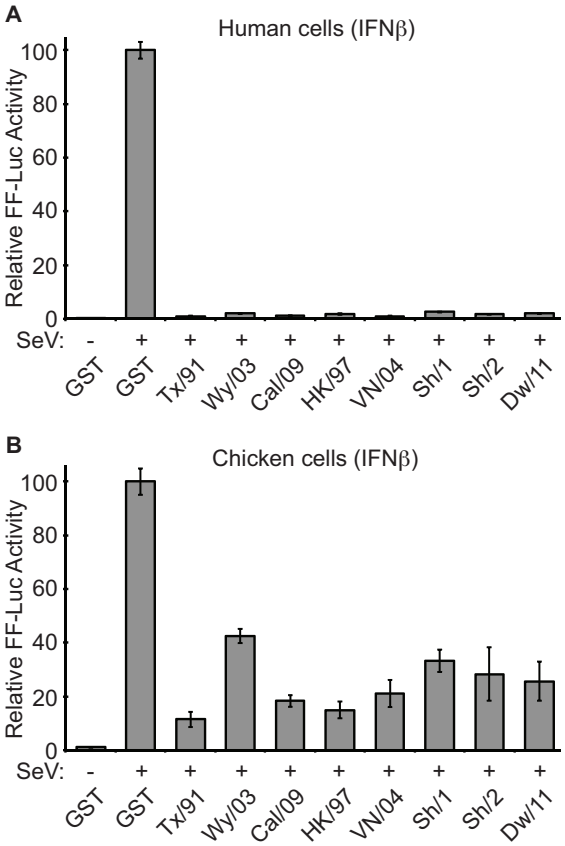

Figure 2.

A

Virus	Strain in this study	NS1 amino-acid 103	106	108	125	189	CPSF30 binding	Gain-of-function phenotype	Reference
Seasonal H1N1 (pre-2009)	Tx/91	F	M	K	D	D	Strong	n/a	n/a
Seasonal H3N2	Wy/03	F	M	K	**E**	D	Strong*	n/a	n/a
Pandemic H1N1 (seasonal 2009+)	Cal/09	F	M	**R**	**E**	**G**	Weak	**Decreased** virulence	Hale *et al.*, JVI, 2010
H5N1 (pre-2004)	HK/97	**L**	**I**	K	D	D	Weak	**Increased** virulence	Spesock *et al.*, JVI, 2011
H5N1 (post-2004)	VN/04	F	M	K	D	D	Strong	n/a	n/a
H7N9	Sh/1	**L**	**I**	K	D	D	Weak	**Increased** virulence	This study

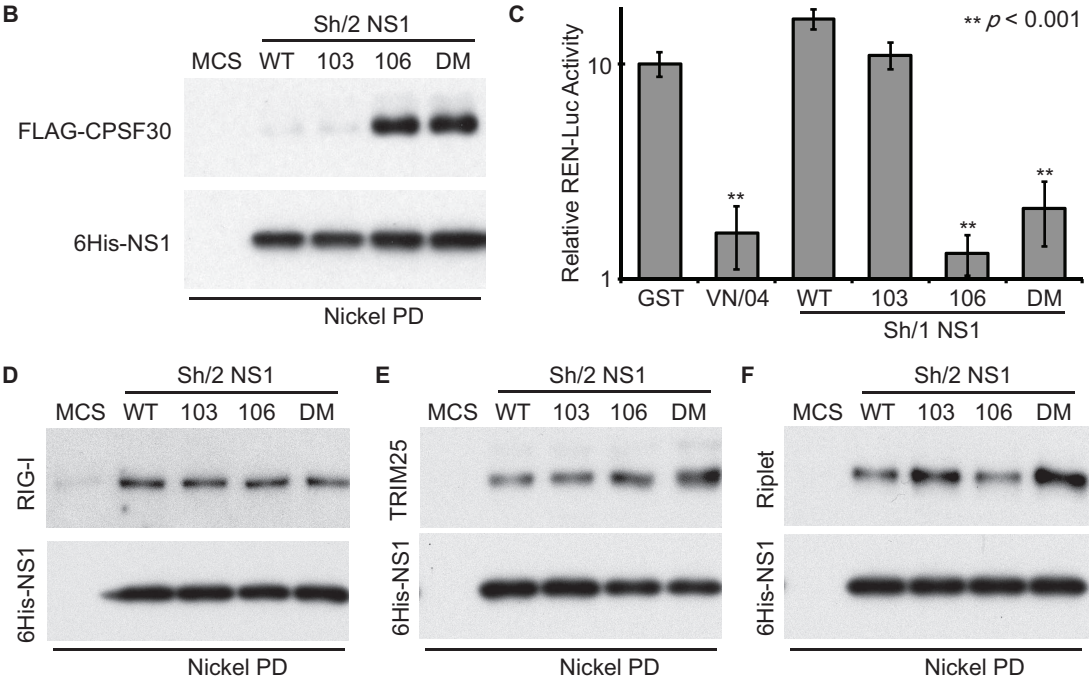

Figure 3.

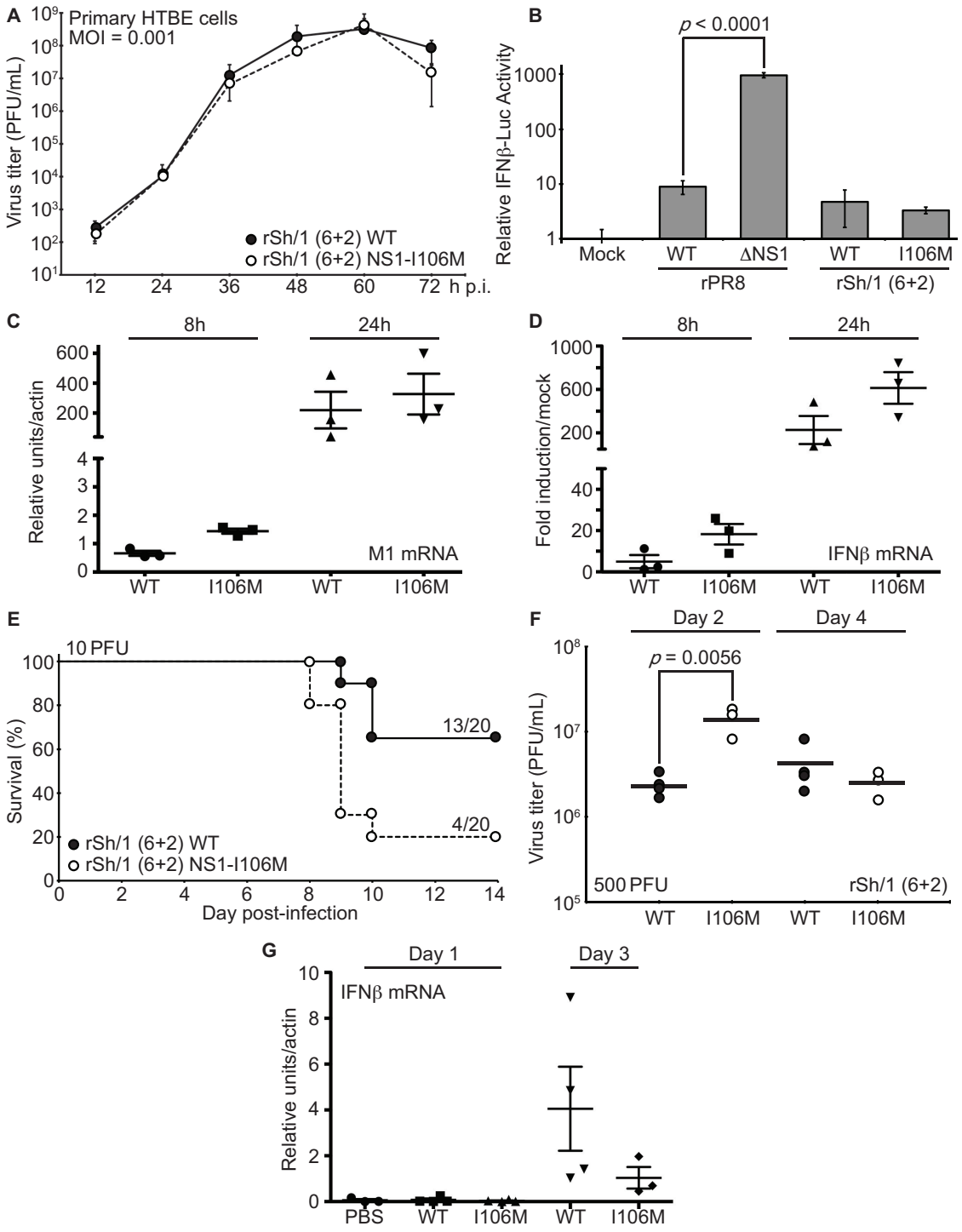

Table 1. Characterization of H7N9-based NS1-WT and NS1-I106M viruses *in vivo*

Virus [rSh/1 (6+2)]	MLD50 [PFU]	Dose [PFU]	Survival [%] (no. of survivors/total no. tested)	Median day of death (range)	Mean maximum wt loss [%] (range)	Median no. of days with indicated wt loss (range) >10%	>20%
		2	100 (15/15)	n/a	8.1 (0.4 - 17.4)	0 (0 - 3)	0 (0)
		10	65 (13/20)	11 (10 - 11)	20.5 (2.5 - >25)	5 (0 - 8)	2 (0 - 7)
NS1-WT	**10.8**	50	15 (3/20)	9 (8 - 10)	24.6 (19.5 - >25)	10 (4 - 10)	8 (0 - 9)
		250	0 (0/5)	9 (8 - 9)	>25 (>25)	10 (10 - 11)	9 (9)
		1250	0 (0/5)	7 (7 - 8)	>25 (>25)	12 (11 - 12)	10 (9 - 10)
		2	100 (15/15)	n/a	13.5 (1.3 - 21.8)	2 (0 - 4)	0 (0 - 2)
		10	20 (4/20)	10 (9 - 11)	22.8 (2.2 - >25)	8.5 (0 - 10)	7 (0 - 8)
NS1-I106M	**4.22**	50	0 (0/20)	8 (8 - 9)	>25 (>25)	10 (10 - 11)	9 (8 - 9)
		250	0 (4/4)	7.5 (7 - 8)	>25 (>25)	11 (11)	9.5 (9 - 10)
		1250	0 (5/5)	7 (6 - 7)	>25 (>25)	12 (11 - 12)	10 (10 - 11)