



Short communication

Two amino acid residues in ion channel protein M2 and polymerase protein PA contribute to replication difference of H5N1 influenza viruses in mice



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ABSTRACT

A/swine/Fujian/1/2001 (FJ1) and A/Duck/Zhejiang/52/2000 (DK52) are H5N1 influenza viruses that are lethal in chickens. However, in mice, FJ1 is highly pathogenic, whereas DK52 cannot replicate at all. In this study, we used reverse genetics to demonstrate that amino acid residues at position 54 of polymerase acidic protein (PA) and position 26 of ion channel protein M2 of FJ1 and DK52 are important determinants for replication in mice. In addition, we prove that M2 and PA proteins contribute to the replication of H5N1 viruses in mice.

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1. Introduction

H5N1 avian influenza viruses have been isolated in wild birds (Chen et al., 2006) and poultry in many countries throughout Asia, Europe, and Africa (<http://www.oie.int>). The molecular basis of the lethality of H5N1 avian influenza viruses in mammalian hosts is still largely unknown, although previous studies have reported that a basic amino acid stretch within the HA protein cleavage site (Hatta et al., 2001), as well as specific amino acids in PB2 (Hatta et al., 2001; Li et al., 2005) and NS1 (Jiao et al., 2008; Seo et al., 2002), contributes to the lethality and host range of H5N1 viruses in mice and pigs. A series of H5N1 influenza viruses isolated from healthy ducks and sporadically sick pigs in southern China in 1999 through 2002 exhibited that the viruses form four pathotypes on the basis of their replication and lethality in mice. A clear temporal pattern can be observed in the progressively increasing pathogenicity of these isolates in the mammalian model (Chen et al., 2004). Two viruses, A/swine/Fujian/1/2001 (FJ1) and A/Duck/Zhejiang/52/2000 (DK52), are similar in genome but display very different replications in mice, although they are both highly lethal in chickens (Chen

et al., 2004). FJ1 can cause the death of mice through lung infection (MLD₅₀, 5.7 LgEID₅₀) (Zhu et al., 2008), whereas DK52 cannot replicate in mice at all (Chen et al., 2004). In the present study, the molecular basis of the replication of these two H5N1 viruses was investigated.

2. Materials and methods

2.1. Materials

Human embryonic kidney cells (293T) were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and incubated at 37 °C in 5% CO₂. FJ1 was isolated from sick pigs with influenza-like symptoms in Fujian province in 2001, and DK52 was isolated from ducks in Zhejiang province in 2000. Virus stocks were propagated in 10-day-old specific pathogen-free (SPF) embryonated chicken eggs and stored at –70 °C until use for RNA extraction and animal study.

2.2. Construction of plasmids

The construction of plasmids for virus rescue was performed as previously described (Li et al., 2005). Mutations were introduced

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into the PA and M2 genes by site-directed mutagenesis (Invitrogen) with a designed set of primers (sequences available upon request).

2.3. Generation of reverse genetic reassortant viruses

Reassortment viruses were generated by reverse genetics as previously described (Hatta et al., 2001; Li et al., 2005). The rescued viruses were detected by hemagglutination assay, and their RNA was extracted and amplified by reverse transcription-PCR (RT-PCR). Each viral segment was sequenced to confirm the identity of the reassortment viruses. The sequence data of the two viruses used in these studies are available in GenBank (Accession Nos. AY747617–AY747623, AY585524, AY585503, AY585482, AY585377, AY585419, AY585440, AY585461, AY585397).

2.4. Animal experiments

For the mouse study, two groups of ten 6-week-old female BALB/c mice (Beijing Experimental Animal Center) were lightly anesthetized with CO₂. 50 µl of H5N1 influenza virus with 50% embryo infectious dose (EID₅₀) of 10^{6.0} and 10^{5.0}, respectively, was inoculated intranasally in each mouse. Three mice in each group were euthanized 3 days post inoculation (p.i.), and 5 p.i., respectively. Organs were collected and titrated for virus infectivity in eggs as previously described (Chen et al., 2004). The remaining mice were monitored for 14 days for weight loss and mortality.

Table 1
Replication of viruses in mice.^a

Virus	Virus titers in organs (log ₁₀ EID ₅₀ ± SD)			
	Inoculated intranasally with 10 ⁶ EID ₅₀		Inoculated intranasally with 10 ⁵ EID ₅₀	
	On day 3 p.i.		On day 3 p.i.	
	Lung		Lung	
FJ1	4.71 ± 0.14	4.71 ± 0.15	4.70 ± 0.12	4.70 ± 0.17
RFJ1	4.71 ± 0.13	4.71 ± 0.18	4.70 ± 0.14	4.70 ± 0.11
RFJ1-DK52PB2	4.61 ± 0.22	4.63 ± 0.26	4.60 ± 0.12	4.60 ± 0.23
RFJ1-DK52PB1	4.63 ± 0.38	4.64 ± 0.33	4.61 ± 0.17	4.61 ± 0.27
RFJ1-DK52PA	1.5 ± 0.11 ^b	1.5 ± 0.13 ^b	1.5 ± 0.12 ^b	1.5 ± 0.14 ^b
RFJ1-DK52HA	4.67 ± 0.22	4.68 ± 0.21	4.66 ± 0.32	4.66 ± 0.24
RFJ1-DK52NA	4.66 ± 0.15	4.67 ± 0.17	4.65 ± 0.13	4.65 ± 0.18
RFJ1-DK52NP	4.68 ± 0.12 ^b	4.69 ± 0.11 ^b	4.67 ± 0.11 ^b	4.67 ± 0.13 ^b
RFJ1-DK52NS	4.62 ± 0.13	4.63 ± 0.12	4.61 ± 0.14	4.60 ± 0.17
RFJ1-DK52M	1.5 ± 0.12 ^b	1.5 ± 0.11 ^b	1.5 ± 0.13 ^b	1.5 ± 0.14 ^b
DK52	–	–	–	–
RD52	–	–	–	–
RD52-FJ1PB2	–	–	–	–
RD52-FJ1PB1	–	–	–	–
RD52-FJ1PA	1.5 ± 0.11 ^b	1.5 ± 0.13 ^b	1.5 ± 0.14 ^b	1.5 ± 0.12 ^b
RD52-FJ1HA	–	–	–	–
RD52-FJ1NA	–	–	–	–
RD52-FJ1NP	–	–	–	–
RD52-FJ1NS	–	–	–	–
RD52-FJ1M	1.5 ± 0.12 ^c	1.5 ± 0.14 ^c	1.5 ± 0.15 ^c	1.5 ± 0.11 ^c
RFJ1PA I54V	1.5 ± 0.12 ^b	1.5 ± 0.13 ^b	1.5 ± 0.13 ^b	1.5 ± 0.14 ^b
DK52PA V54I	1.5 ± 0.11 ^c	1.5 ± 0.12 ^c	1.5 ± 0.16 ^c	1.5 ± 0.13 ^c
FJ1PA C384Y	4.68 ± 0.17	4.68 ± 0.18	4.66 ± 0.21	4.67 ± 0.14
DK52PA Y384C	–	–	–	–
FJ1PA V459I	4.67 ± 0.14	4.67 ± 0.17	4.63 ± 0.16	4.64 ± 0.15
DK52PA I459V	–	–	–	–
FJ1PA V330I	4.67 ± 0.20	4.68 ± 0.14	4.66 ± 0.23	4.65 ± 0.18
DK52PA I330V	–	–	–	–
RFJ1M2S26L	1.5 ± 0.13 ^b	1.5 ± 0.12 ^b	1.5 ± 0.14 ^b	1.5 ± 0.16 ^b
DK52M2L26S	1.5 ± 0.14 ^c	1.5 ± 0.13 ^c	1.5 ± 0.17 ^c	1.5 ± 0.12 ^c
FJ1PA I54V M2 S26L	–	–	–	–
DK52 PA V54I M2 L26S	2.5 ± 0.13 ^c	2.5 ± 0.11 ^c	2.5 ± 0.12 ^c	2.5 ± 0.16 ^c

^a Six-week-old SPF BALB/c mice were inoculated intranasally with 10⁶ EID₅₀ and 10⁵ EID₅₀ doses of each virus in a 50-µl volume, respectively. Three mice from each group were killed on days 3 p.i., and on days 5 p.i., respectively. Organs were then collected for virus titration in eggs. (–) No virus was detected.

^b P value was <0.01 compared with the titers in the corresponding organs of the FJ1-inoculated mice.

^c P value was <0.01 compared with the titers in the corresponding organs of the DK52-inoculated mice.

The mouse studies were approved by the Review Board of Shandong Veterinary Research Institute, Shandong Academy of Agricultural Sciences.

3. Results and discussion

After examining all the PA and M2 sequences of H5N1 subtype influenza virus in GenBank, we found that 1049 influenza viruses contained the amino acid Ile at position 54 of the PA protein. Six influenza viruses possessed Val at the same position, including A/Viet Nam/DT-036/2005, A/Viet Nam/LA-028/2005, A/Viet Nam/VL-020/2005, A/Vietnam/CL2009/2005, A/Vietnam/CL115/2005 and A/Duck/Zhejiang/52/00. A total of 1152 viruses had the amino acid Leu at position 26 of the M2 protein, whereas FJ1 possessed Ser at the same position. The results indicate that amino acids Ile and Leu are typically found at position 54 of the PA protein and at position 26 of the M2 protein of H5N1 subtype influenza viruses, respectively. Few H5N1 influenza viruses contain Val and Ser at the same protein positions. A/Viet Nam/DT-036/2005, A/Viet Nam/LA-028/2005, A/Viet Nam/VL-020/2005, A/Vietnam/CL2009/2005, and A/Vietnam/CL115/2005 are pathogenic to humans.

We cloned the cDNA of each full-length RNA segment of FJ1 and DK52 viruses into a vRNA–mRNA bi-directional expression plasmid pBD (Li et al., 2005) using a set of gene segment-specific primers (sequences available upon request) as previously described (Liet al., 2005). Using these plasmids, we rescued the FJ1 and DK52 viruses,

designated as RFJ1 and RDK52, and grew them in 10-day-old SPF embryonated chicken eggs. We also tested their replication in mice. As shown in Table 1, the rescued RDK52, similar to the wild-type DK52, was unable to replicate in any organ of the mice (Table 1). In

this group, no mouse died, and all of them exhibited 10% increase in body weight by day 14 p.i. (data not provided). By contrast, the RFJ1 virus replicated in the lungs of the mice without adaptation, and the mean titer reached 4.71 LgEID₅₀ on day 3 p.i. However,

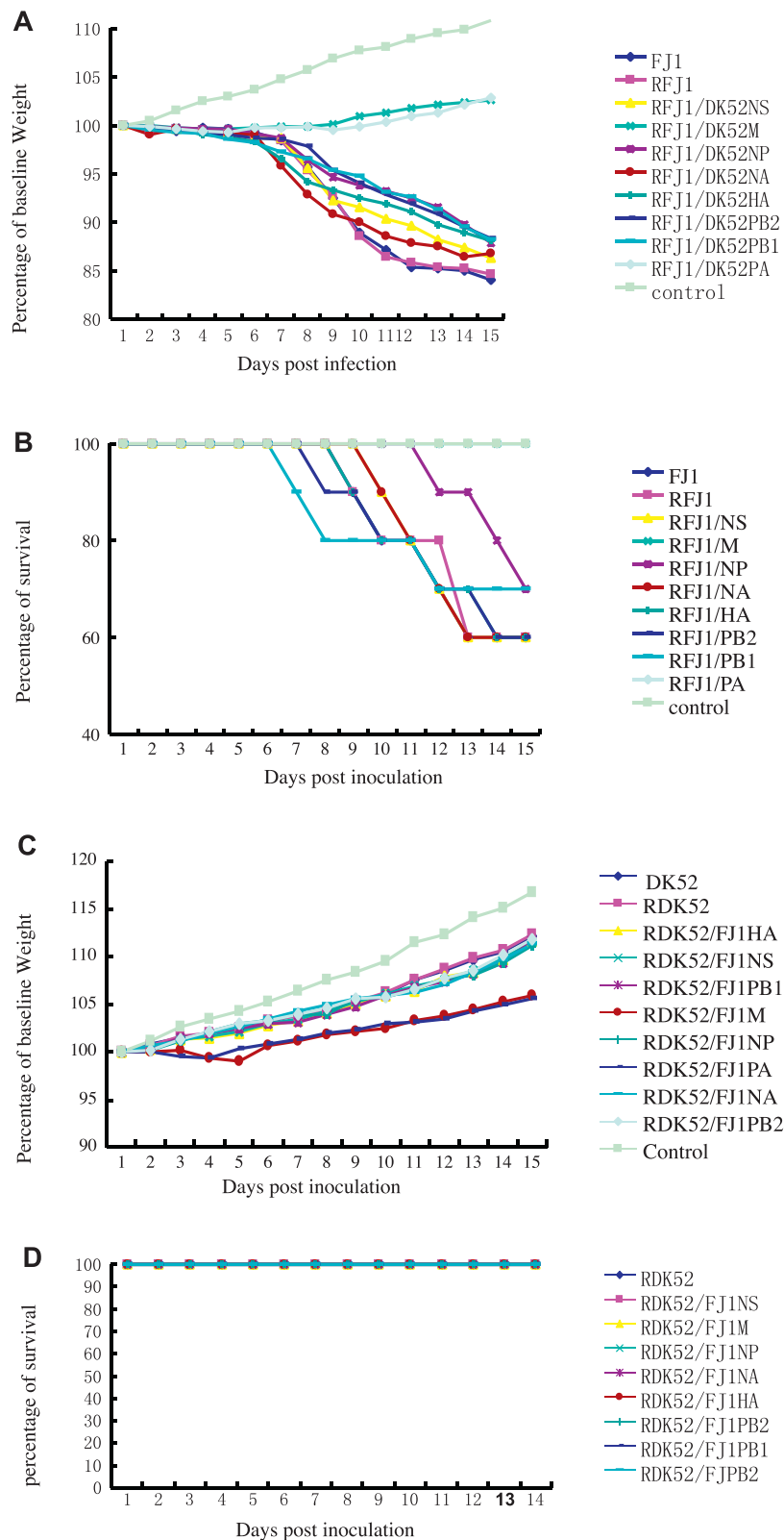


Fig. 1. Comparison of weight changes and death patterns of the mice infected with different H5N1 influenza reassortment viruses. Mice (10 mice/group) were intranasally infected with 10^6 EID₅₀ of virus. (A) and (B) Mice infected with RFJ1 and the reassortants in the FJ1 background. (C) and (D) Mice infected with RDK52 and the reassortants in the DK52 background.

its replication was restricted to the lungs, a performance that is similar to that of the wild-type FJ1 (Table 1). By day 14 p.i., the RFJ1 virus killed six mice and caused over 15% body weight loss to the remaining ones (data not provided). Viruses isolated from dead mice were fully sequenced to ensure the absence of unwanted mutations.

FJ1 and DK52 differ by only 23 amino acids in their genome (Fig. 2). To identify the genes that contribute to replication in mice, we generated eight single-gene reassortants as previously described (Hatta et al., 2001; Li et al., 2005), each containing one gene derived from DK52 and seven from FJ1. We tested their replication in mice as previously described by Chen et al. (2004). The recombinant viruses that contained the PB2, PB1, HA, NA, NP, and NS genes of DK52 (designated as RFJ1-DK52PB2, RFJ1-DK52PB1, RFJ1-DK52HA, RFJ1-DK52NA, RFJ1-DK52NP, and RFJ1-DK52NS, respectively) displayed the same replication in mice as the wild-type FJ1 virus, and caused mouse weight loss (Fig. 1A) and death at different degrees (Fig. 1B). None of the viruses was detected in the spleen, kidney, and brain, as they only replicated in the lungs of the mice (Table 1). The RFJ1-DK52 PA and RFJ1-DK52M viruses did not cause death and only replicated in the lungs of mice (virus titers: 1.5 LgEID₅₀ and 1.5 LgEID₅₀, respectively) (Table 1). The RFJ1-DK52PA and RFJ1-DK52M viruses were attenuated 10³-fold higher than that of the wild-type FJ1 virus (virus titers: 1.5 LgEID₅₀ vs. 4.71 LgEID₅₀).

The effect of individual genes derived from FJ1 on the replication of DK52 was tested. We generated eight single-gene reassortant viruses, each bearing one gene from FJ1 and the remainder from DK52. The viruses that carried the PB2, PB1, HA, NA, NS, and NP genes of FJ1 (RDK52-FJ1PB2, RDK52-FJ1PB1, RDK52-FJ1HA, RDK52-FJ1NA, RDK52-FJ1NP, and RDK52-FJ1NS, respectively) were similar to the wild-type DK52, that is, they did not replicate in any organ tested (Table 1). Moreover, they did not cause mouse death and weight loss (Fig. 1C and D). However, RDK52-FJ1PA and RDK52-FJ1M were able to replicate, and their virus titers in the lungs of mice inoculated with their aliquots were very low (virus titers: 1.5 LgEID₅₀ and 1.5 LgEID₅₀, respectively) (Table 1).

The introduction of the PA and M genes of the DK52 virus remarkably attenuated the replication ability of FJ1 by over 1000-fold. Compared with the virus titer of FJ1 (4.71 log₁₀ EID₅₀), those of FJ1-DK52 PA or FJ1-DK52M were lower (1.5 log₁₀ EID₅₀). The reassortant virus did not cause death to the mice. The results gathered from these reassortant virus experiments indicate that multiple genes from the DK52 virus attenuate the replication ability of the FJ1 virus in mice, whereas the PA and M genes contribute more to the virulence difference of the two viruses (Table 1). We focused on the PA and M genes for further analyses.

The PA proteins of the FJ1 and DK52 viruses differ by four amino acids, and their M proteins have one amino acid difference in the M2 protein (Fig. 2). To elucidate the molecular basis of the replication discrepancy between the FJ1 and DK52 viruses, we generated 10 mutant viruses, each containing a substitution on the PA (at positions 54, 330, 384, and 459) or M2 (position 26) amino acid residues. The effects of the mutant viruses were tested for their replication in mice (Table 1). FJ1PAV330I, FJ1PAC384Y, FJ1PAI459V, DK52PAI330V, DK52PAY384C, and DK52PAV459I retained the characteristics of their wild-type sources (Table 1). However, FJ1PAI54V, FJ1M2S26L, DK52PAV54I, and DK52M2 L26S exhibited similar replication in mice as the single gene substitution in the PA or M genes of the viruses (Table 1). These results indicate that the amino acids at position 54 of PA and position 26 of M2 are responsible for the difference in the replication of the FJ1 and DK52 viruses in mice.

Two mutants encoding double mutations at position 54 of PA and position 26 of M2 (FJ1PAI54V M2 S26L, DK52 PAV54I M2 L26S) were generated and tested for their replication in mice. FJ1

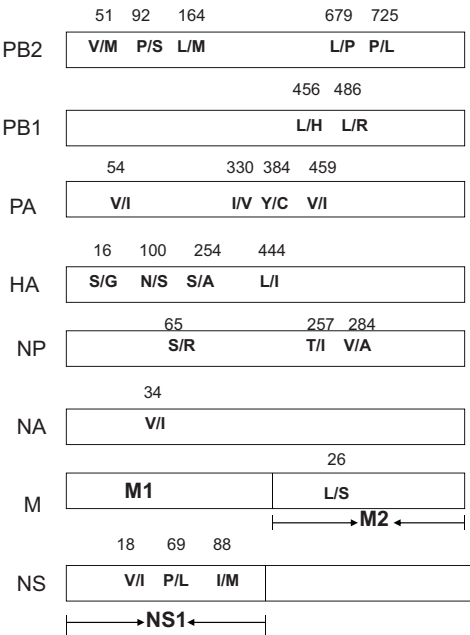


Fig. 2. Amino acid differences between the FJ1 and DK52 viruses. The amino acid differences between the two viruses are shown as single letters: DK52 on the left of the slash and FJ1 on the right.

PAI54V M2 S26L was unable to replicate in mice, whereas DK52 PAV54IM2L26S replicated in the lungs of the mice (virus titer: 2.5 LgEID₅₀) (Fig. 3). This result indicates that substitutions at two amino acid locations (position 54 of PA and position 26 of M2 gene) can completely change the replication behavior of FJ1 and DK52 viruses in mice.

Mutagenesis studies have shown that PA is involved in endonuclease activity (Fodor et al., 2002; Hara et al., 2006) of PB1 (Li et al., 2001). Furthermore, PA is involved in elongation facilitated by polymerases (Fodor et al., 2003), as well as in cap binding, and vRNA and cRNA promoter binding (Hara et al., 2006). A function for PA in the assembly of viruses has also been suggested (Regan et al., 2006), and PA possesses or induces proteolytic activity, the significance of which is not fully understood (Hara et al., 2001; Perales et al., 2000; Rodriguez et al., 2007; Sanz-Ezquerro et al., 1996; Toyoda et al., 2003).

The influenza A virus M2 protein has a pivotal role in mediating viral budding and scission (Rossman et al., 2010). M2 is a 97-amino acid long integral membrane protein that associates into a homotetrameric proton selective ion channel and discharges multiple roles during the infectious cycle of the virus (Wang et al., 2011). Residing in the virus membrane, its sequence encompasses a 19-residue hydrophobic transmembrane domain (TM; residues 25–43). M2 undergoes tetramerization to form a pore, which can function as an ion channel that selectively allows the influx of H⁺ during cell infection and acidification of the virus lumen (Lamb et al., 1985; Pinto et al., 1992; Sakaguchi et al., 1997). Several structures of the M2 channel, elucidated through NMR methods and X-ray crystallography, have been published, mostly by the groups of Chou, Cross, and DeGrado (see Acharya et al., 2010; Pielak and Chou, 2010; Sharma et al., 2010; Wang et al., 2013). Although the published structures are slightly different from one another, they all exhibit the same basic topology of a tetramer composed of tilted helices. Among the most prominent resistance-conferring mutations are the substitutions of Ser31 to Asn (S31N) (Krumholz et al., 2009) and Val27 to Ala (V27A), the former being present in the resistant swine flu strain. Additional mutations in other pore-lining residues, such as Ala30 and (rarely) Gly34 have also

Virus	Other genes	PA	Replication	Tier(lgEID ₅₀)	MLD ₅₀ (lgEI D ₅₀)	
54						
RFJ1	FJ1	I		4.71	5.7	
RDK52	DK52	V		—	6.5	
RFJ1PA I54V	FJ1	V		1.5	6.1	
RDK52V 54I	DK52	I		1.5	6.1	
M2						
26						
RFJ1	FJ1	S		4.71	5.7	
RDK52	DK52	L		—	6.5	
RFJ1M2S26L	FJ1	L		1.5	6.1	
RDK52L26S	DK52	S		1.5	61	
PA M2						
54 26						
RFJ1PAI54VM2S26L	FJ1	V	L		—	6.5
RDK52PAV 54IM2L26S	DK52	I	S		2.5	5.9

Fig. 3. PA and M2 mutant viruses and their virulences in mice. The color of the bar indicates the origin of the gene as follows: blue, DK52; red, FJ1. The corresponding amino acids are shown as single-letter abbreviations with the positions numbered at the top. The red dots in the mouse figures indicate tissue tropism (lower left, lung). The mutated amino acids are shown in red or blue and italics. Amino acid abbreviations: I, Ile; V, Val; S, Ser; L, Leu. Six-week-old SPF BALB/c mice were inoculated intranasally with 10^6 EID₅₀ of each mutant virus in a 50- μ l volume, respectively. Three mice from each group were killed on days 3 p.i., and organs were collected and titrated for virus infectivity in eggs as described previously (Chen et al., 2004). (–) No virus was detected. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

been reported as resistance conferring (Hay et al., 1985; Sweet et al., 1997). Previous research has suggested that these mutations cause changes in the pore radius, resulting in their interference with the binding of drugs (Leonov et al., 2011).

In our study, we found that amino acid substitutions at position 54 of PA and position 26 of M2 are critical to the replication of FJ1 and DK52 viruses in mice. This report is the first to present the involvement of M2 in the replication behavior of H5N1 influenza in mice, although the underlying molecular mechanism remains unknown.

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