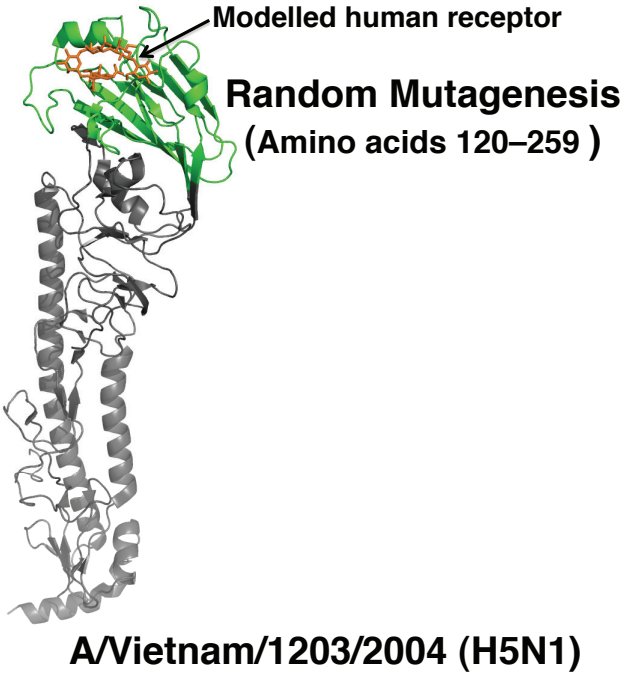
doi:10.1038/nature10831

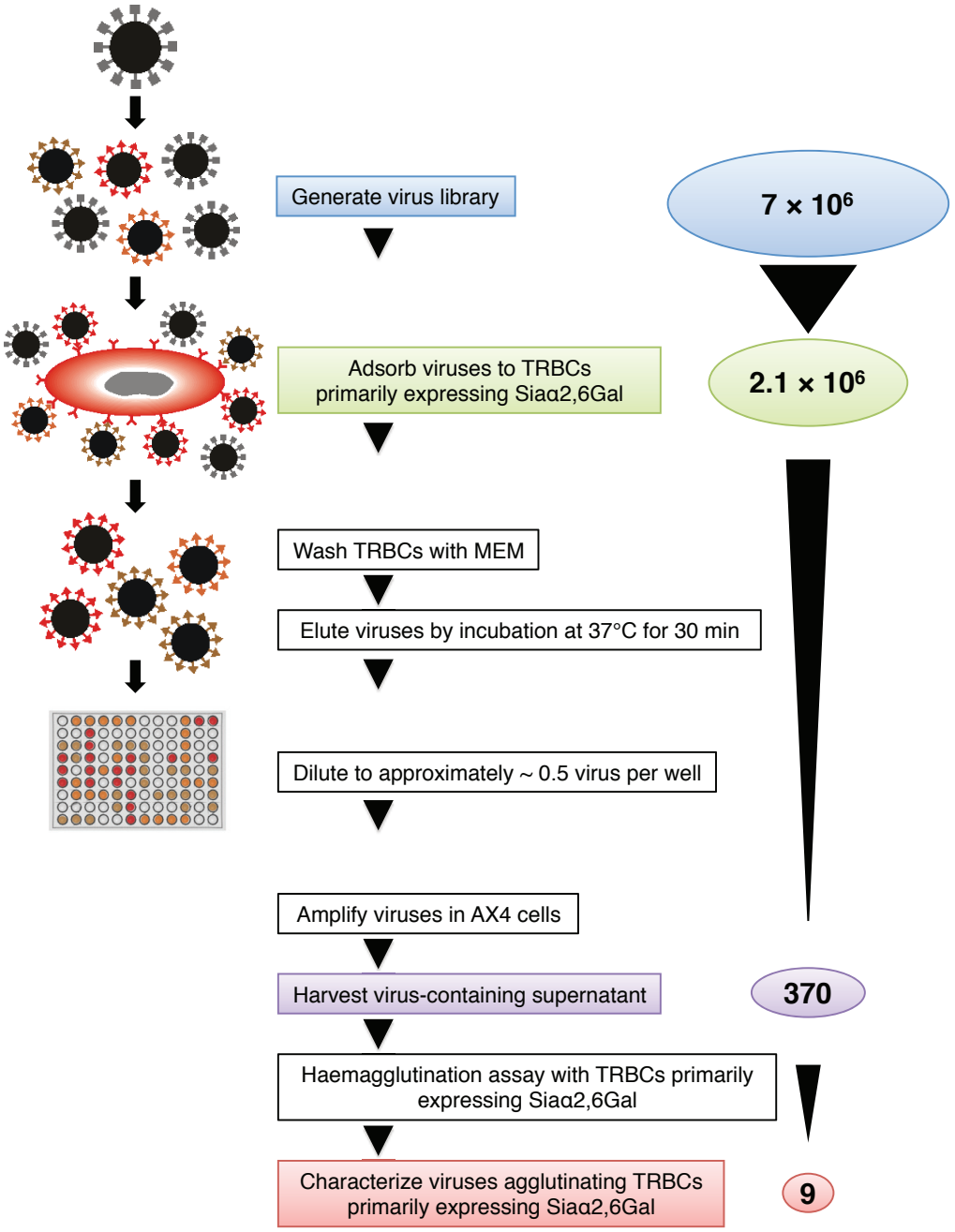
1. Pathological analyses of H5 avian-human reassortant viruses.

The rgCA04 infection in the lungs of ferrets mainly caused severe bronchopneumonia with extensive viral antigen expression in the peribronchial glands, terminal bronchioles, and a few alveolar cells (**Supplementary Fig. 10**), as was reported previously[1]. On the other hand, the rgVN1203/CA04 virus caused mainly alveolitis in a few lung lobes without involvement of the peribronchial glands. Although no significant differences in the lung lesions were detected among rgCA04, rg(N224K/Q226L)/CA04, and HA(N158D/N224K/Q226L)/CA04 viruses, the lung lesions caused by rgVN1203/CA04 and HA(N158D/N224K/Q226L/T318I)/CA04 were less severe than those caused by CA04 on day 6 after infection (Dunnett's test; P=0.008 and 0.02, respectively; **Supplementary Fig. 11**). Collectively, these findings suggest that the HA mutations affect tissue tropism (i.e., replication in nasal mucosa) and the extent of lung injury. Interestingly, the H5 avian-human reassortants containing a multibasic amino acid motif at the HA cleavage site did not cause extensive systemic infection or death in ferrets, although we did detect virus in some non-respiratory organs.

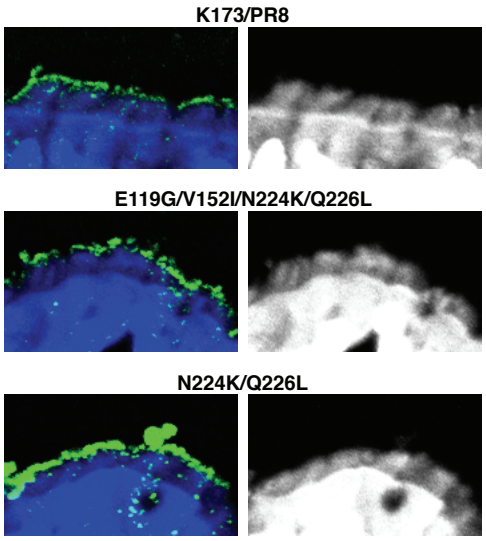
2. Supplementary figures



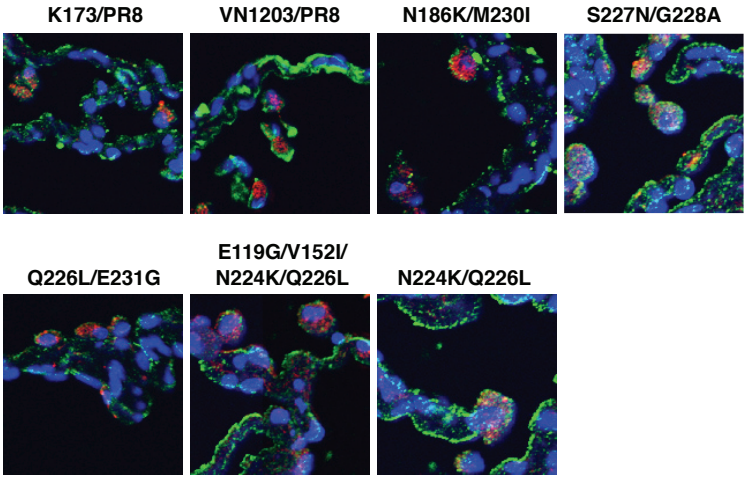
Supplementary Figure 1: Three-dimensional structure of the monomer of VN1203 HA [Protein Data Bank (PDB) ID: 2FK0][2]. The region mutated in this study is shown in green. The human receptor analog [derived from its complex with H9 HA (PDB ID: 1JSI)[3]] is docked into the structure (shown in orange). Images were created with MacPymol [http://www.pymol.org/].



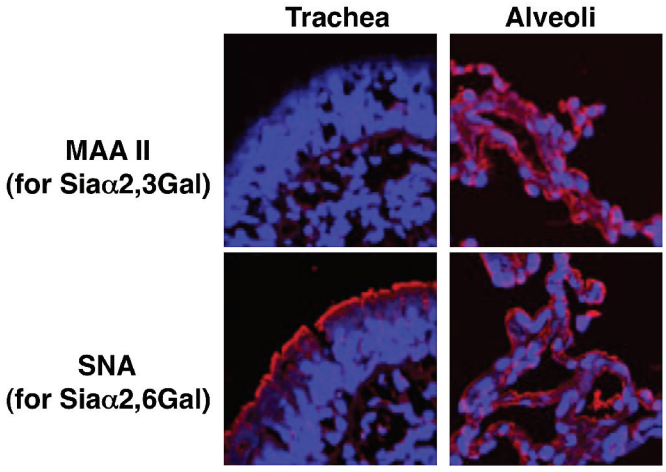
Supplementary Figure 2: Schematic overview of library screening. A virus library possessing HA proteins with random mutations in the head portion was adsorbed at 4°C to turkey red blood cells (TRBCs) treated with an α2,3-linkage-specific sialidase. Unbound viruses were removed by extensive washing, and bound viruses were eluted by incubation at 37°C. Eluted viruses were amplified in AX4 cells (MDCK cells modified to overexpress Siaα2,6Gal), and rescreened for the ability to agglutinate α2,3-sialidase-treated TRBC. From a library of 7 x 10^6 viruses, 2.1 x 10^6 viruses were screened in three independent experiments (with 0.7 x 10^6 viruses per experiment). We isolated 370 viruses, nine of which agglutinated α2,3-sialidase-treated TRBC.



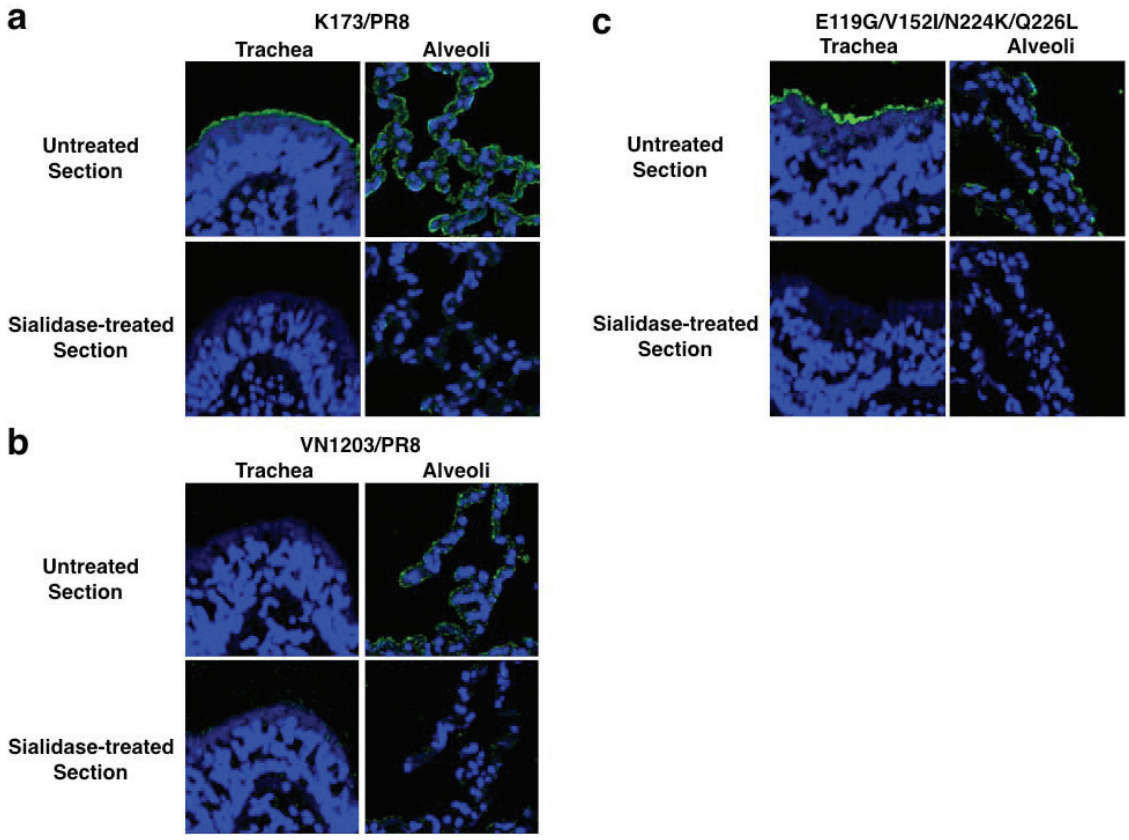
Supplementary Figure 3: The attachment of virus to ciliated cells in human tracheal epithelium. Tracheal sections from **Fig. 2c** are shown at higher magnifications (left panels). Right panels in black and white show ciliated cells stained with Hoechst dye in the same field as that on the left.



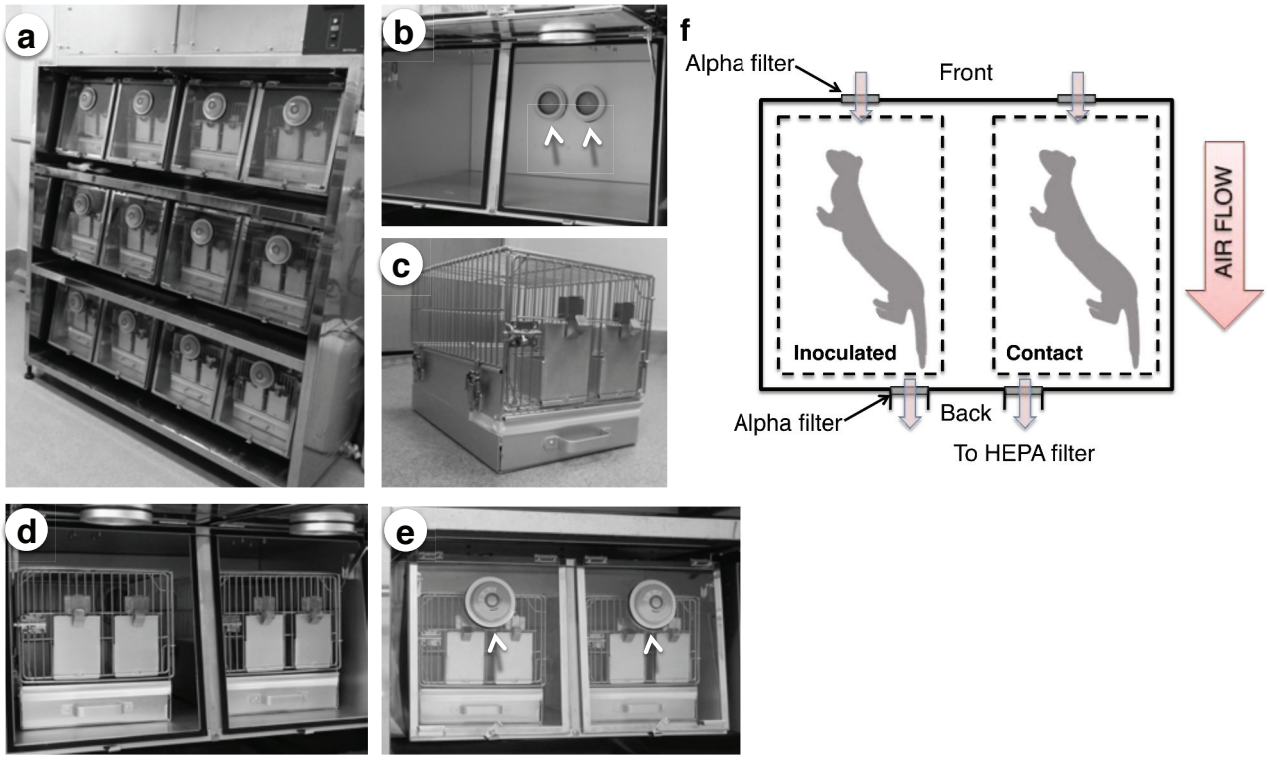
Supplementary Figure 4: Virus binding to type II pneumocytes of human lung tissue. Type II pneumocytes were detected by using immunofluorescence staining for surfactant protein A (red). Virus attachment (green) to the human lung tissue was observed as described in **Fig. 2c**.



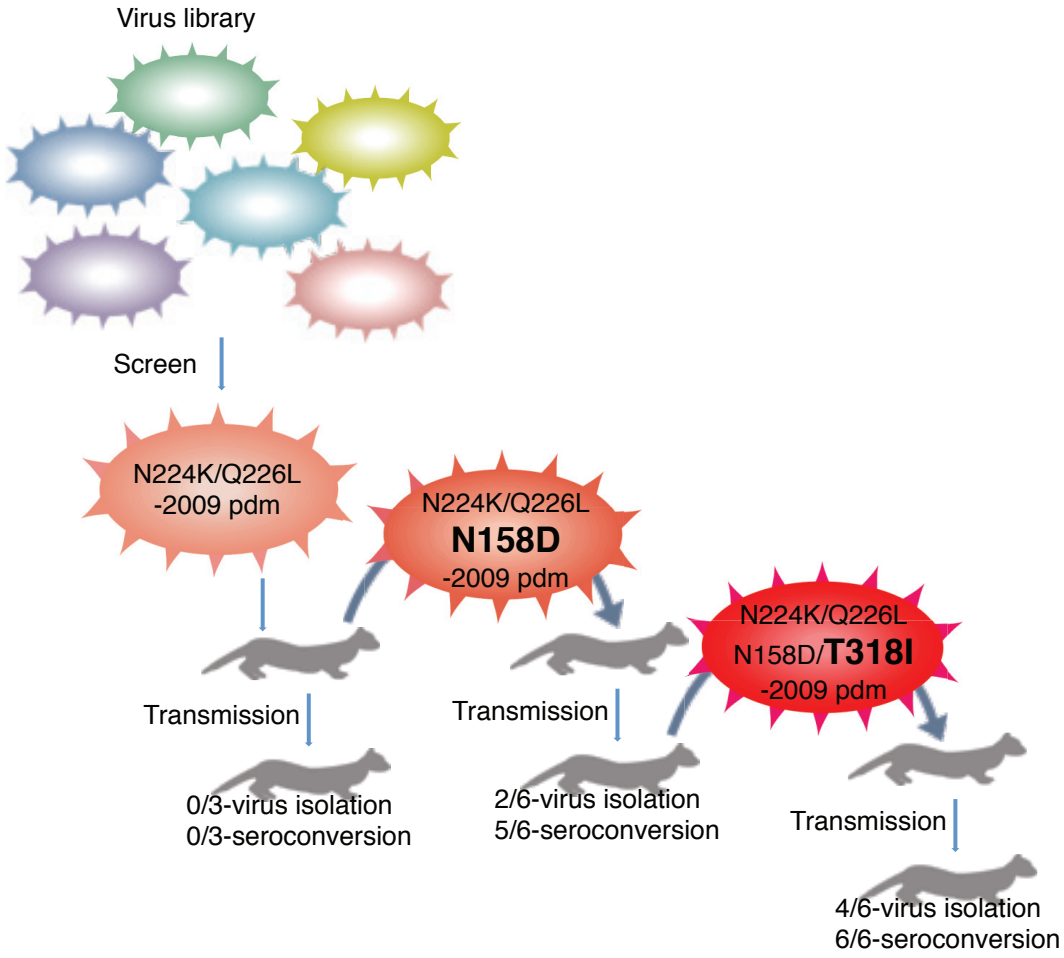
Supplementary Figure 5: Detection of Siaα2,3Gal and Siaα2,6Gal receptors in the human respiratory tract. Sections of human tracheal and alveolar tissues were stained with either biotinylated *Maackia amurensis* (MAA) II for Siaα2,3Gal (red) (upper panel) or biotinylated *Sambucus nigra* (SNA) for Siaα2,6Gal (red) (lower panel); nuclei were visualized with Hoechst dye (blue). In the trachea, Siaα2,6Gal receptors are mainly expressed on the epithelial cells. In alveoli, both Siaα2,6Gal and Siaα2,3Gal receptors are detected on the epithelial cells.



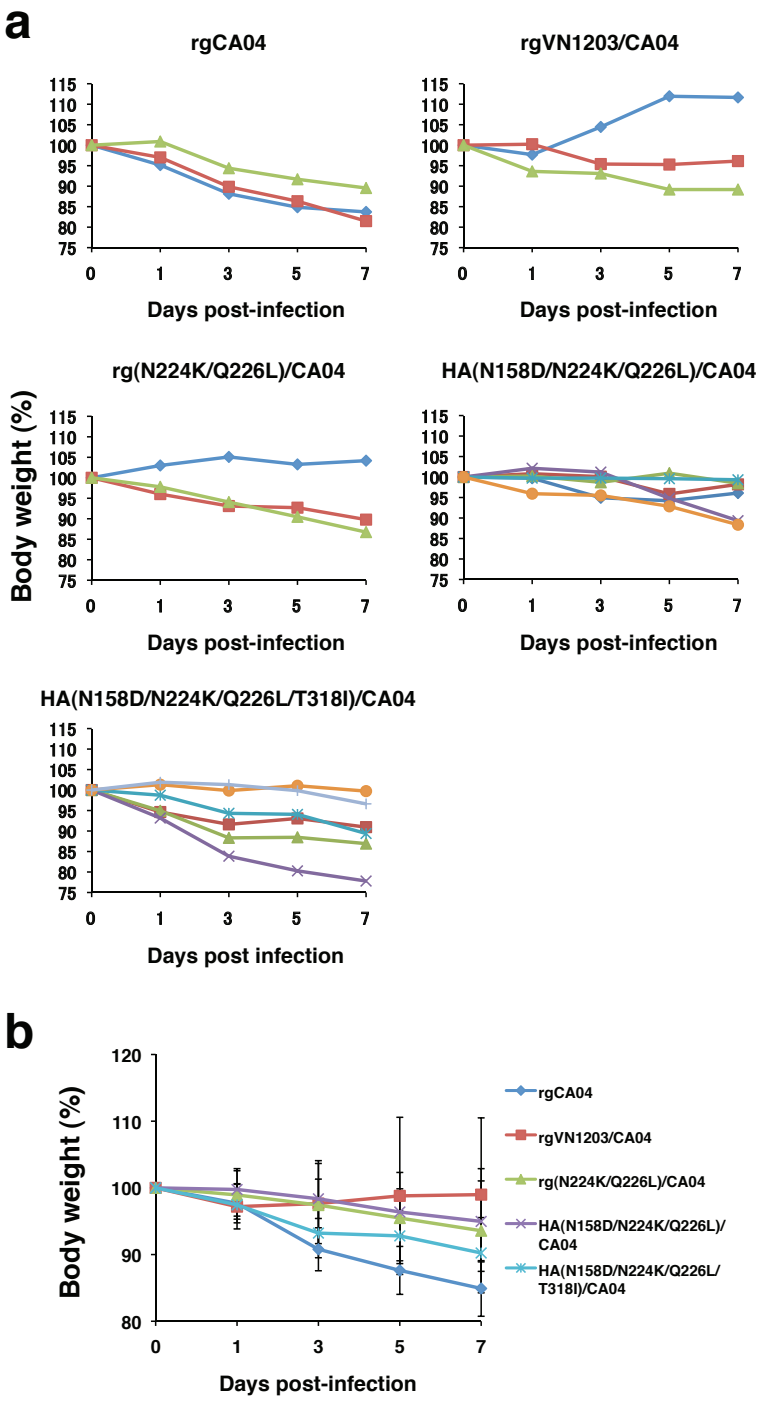
Supplementary Figure 6: Sialic acid-specific virus binding to human respiratory tissues. Sections of human tracheal and alveolar tissues were treated with *Arthrobacter ureafaciens* sialidase prior to incubation with the K173/PR8 (a), VN1203/PR8 (b), and E119G/V152I/N224K/Q226L (c) viruses. Sections were then incubated with either rabbit anti-K173 polyclonal (green) or mouse anti-VN1203 HA monoclonal antibodies (green), fluorescently labeled secondary antibodies, and Hoechst dye (blue). Virus binding to tissues was reduced substantially following sialidase treatment.



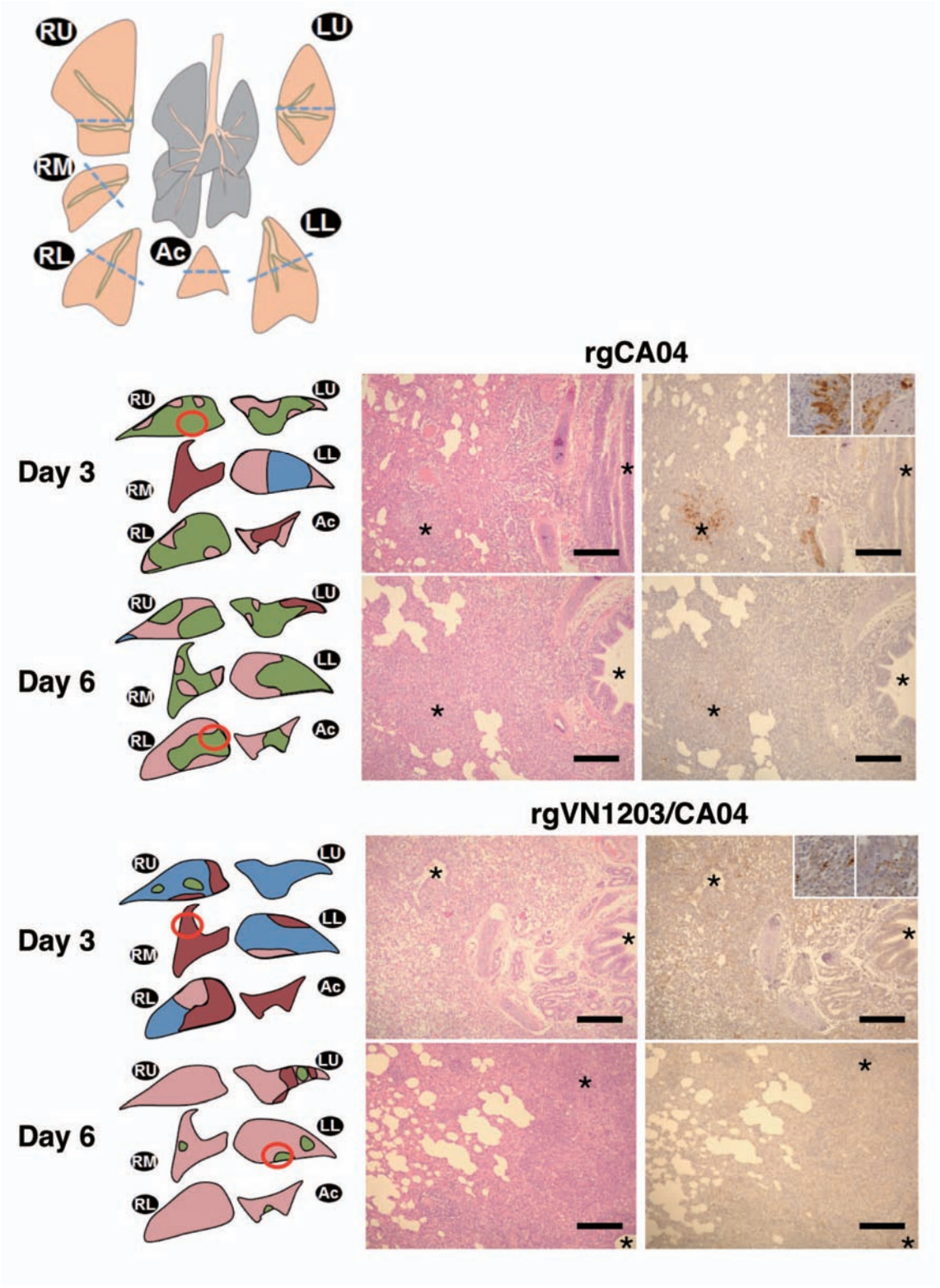
Supplementary Figure 7: System used to study ferret transmission. Photographs (a–e) and a schematic representation (f) of the system used are shown. The isolator (a) (model: S-2176F23W, Showa Science CO., LTD, Japan) was designed for this ferret transmission study. It consists of a main body (W1570 x D700 x H2085 mm) and six carrying boxes (b) (dimensions of each box: W680 x D500 x H350 mm). Air supply and exhaust for each carrying box were processed through alpha-ring filters, which are indicated by arrows (b, e, f), and the volume of exhaust air was 2.5 m^3 per minute. The exhaust air from each carrying box was filtered by a high efficiency particulate air (HEPA) filter to prevent cross-contamination with other carrying boxes placed in the same unit of the isolator. Inoculated (= "inoculated" in f) and naïve (= "contact" in f) ferrets were housed in a "transmission cage", a ferret cage with an open wire (W280 x D380 x H300 mm) (c, f). Two transmission cages were positioned 5 cm apart in the carrying box (d, e; pictures show the carrying boxes with open doors (d) and with closed doors (e). All transmission experiments reported in this study were conducted in an animal biosafety level 3-Agriculture containment laboratory under controlled conditions of temperature (20°C –25°C) and humidity (38.4% ± 8.8%).

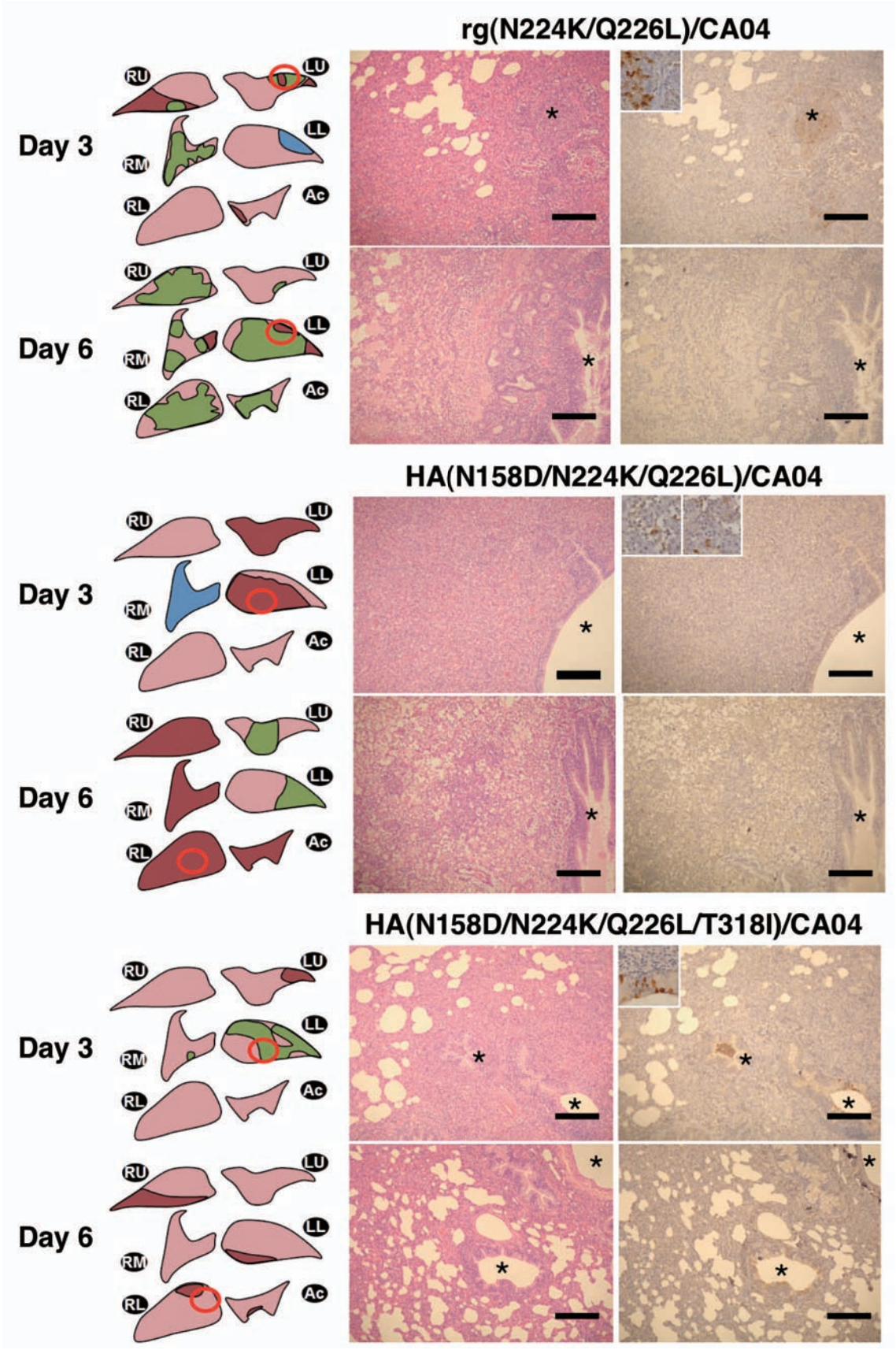


Supplementary Figure 8: Schematic overview of the selection of H5 HA mutants that confer transmissibility in ferrets. A virus library was screened for variants that acquired the ability to bind to human-type receptors. One variant possessing two mutations in HA (N224K/Q226L) bound efficiently to human-type receptors and human respiratory tissues, but did not transmit among ferrets. Replication of this variant in ferrets resulted in a virus possessing an additional mutation in HA (N158D). This virus transmitted in 2 of 6 pairs of ferrets, as detected by virus isolation, and in 5 of 6 pairs, as detected by seroconversion. A virus isolated from one of the contact animals acquired a fourth mutation in HA (T318I). This variant was transmitted in 4 of 6 pairs of ferrets, as detected by virus isolation, and in 6 of 6 pairs, as detected by seroconversion.

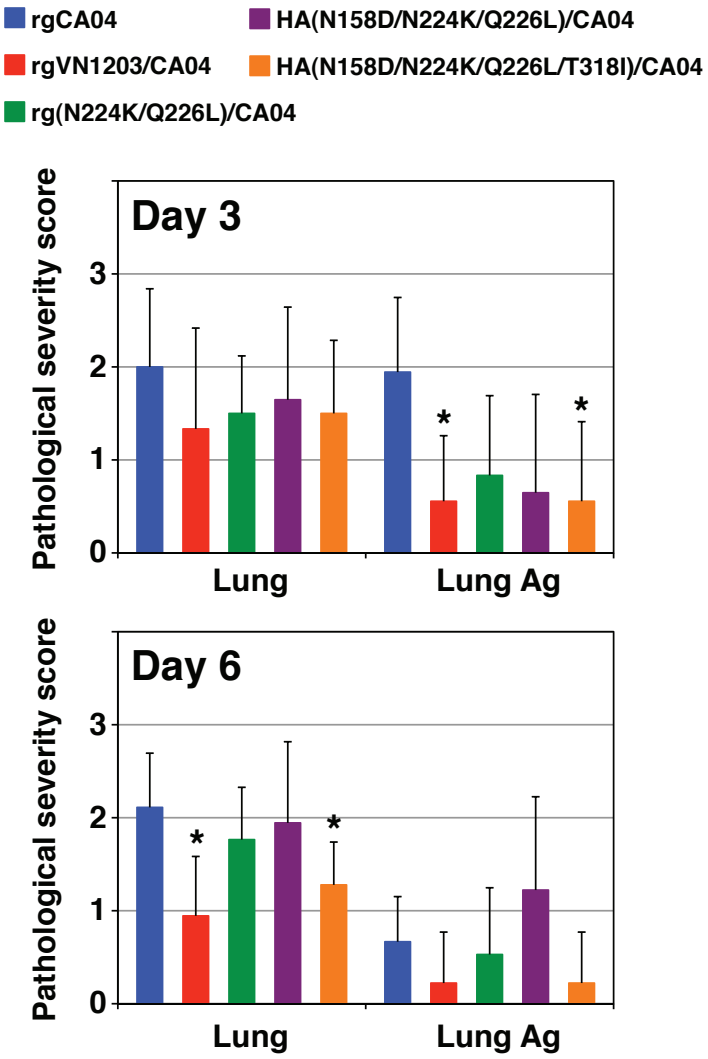


Supplementary Figure 9: Body weight changes in infected ferrets. Ferrets were inoculated intranasally with 10^6 p.f.u. of rgCA04 (n=3), rgVN1203/CA04 (n=3), rg(N224K/Q226L)/CA04 (n=3), HA(N158D/N224K/Q226L)/CA04 (n=6) or HA(N158D/N224K/Q226L/T318I)/CA04 (n=6). **a,** Body weights of individual ferrets inoculated with viruses are depicted as a percentage of body weight compared to those on day 0 after infection. **b,** Percentages of mean body weight and standard deviation are shown.


RU
LU
RM
LL
RL
Ac
rgCA04
Day 3
Day 6
rgVN1203/CA04
Day 3
Day 6


rg(N224K/Q226L)/CA04
Day 3
Day 6
RU
LU
RM
LL
RL
Ac
HA(N158D/N224K/Q226L)/CA04
Day 3
Day 6
HA(N158D/N224K/Q226L/T318I)/CA04
Day 3
Day 6

Supplementary Figure 10: Representative histological changes in lungs from influenza virus-infected ferrets. Three ferrets per group were infected intranasally with 10^6 p.f.u. of virus, and tissues were collected on days 3 and 6 after infection for pathological examination. Left panel, the histological findings in the lungs are represented as schematic diagrams for one of the three animals per group. Cutting aspects of each lung lobe are illustrated with lesion distribution: blue, normal appearance; green, bronchopneumonia; red, alveolitis; and pink, interstitial thickening. Red circles indicate the areas shown in the histology sections. Ac, accessory lobe; LL, left lower lobe; LU, left upper lobe; RL, right lower lobe; RM, right middle lobe; RU, right upper lobe. The middle panel shows haematoxylin and eosin staining; the right panel shows immunohistochemical staining for viral antigen detection (brown staining). Bronchi are indicated by asterisks "*". Inserts show a higher magnification of the antigen-positive cells. The lesions were diagnosed as bronchopneumonia when the lung lesion was observed mainly around the inflammatory or viral antigen-positive bronchi/bronchioles. By contrast, the lesions were diagnosed as alveolitis when bronchi and bronchioles remained intact and inflammation was observed mainly in alveoli. Interstitial thickening was occasionally observed as stimulation by inoculum or secondary to the severe inflammation of adjacent tissue. Bars, 200 μm.



Supplementary Figure 11: Pathological severity scores in infected ferrets. To represent comprehensive histological changes, respiratory tissue slides were evaluated by scoring the pathological changes and viral antigen expression levels. The pathological scores were determined for each animal in each group (n = 3/group on days 3 and 6 pi) using the following scoring system: 0 = no pathological change/antigen negative; 1 = affected area (<30%) or only interstitial lesion/rare viral antigens; 2 = affected area (<80%, ≥30%)/moderate viral antigens; 3 = severe lesion (≥80%)/many viral antigens; Lung: pathologic changes in the lungs, Lung Ag: viral antigens in the lungs. Asterisks indicate virus pathological scores significantly different from that of rgCA04 (Dunnett's test; $P < 0.05$). Error bars denote standard deviation.

3. Supplementary Tables

Supplementary Table 1: Binding of VN1203 variants to turkey red blood cells

	Viruses isolated from library[a]		Recreated viruses[b]	
	Siaα2,3/α2,6-TRBC[c]	Siaα2,6-TRBC[c]	Siaα2,3/α2,6-TRBC	Siaα2,6-TRBC
K173/PR8	≥ 256	≥ 256	512	512
VN1203/PR8	≥ 256	< 2	512	< 2
Q226L/G228S (control)	nd	nd	512	64
N186K/M230I	≥ 256	2	512	2
S227N/G228A	64	16	512	1024
S136N	64	16	nd	nd
I202T/R220S	64	32	512	64
Q226L/E231G	≥ 256	128	512	256
W153R/T160I	8	4	512	32
E119G/V152I/N224K/Q226L	≥ 256	≥ 256	512	512
N169I/H184L/I217M	64	8	512	64
H130Q/K157E	≥ 256	16	512	16

[a]Viruses isolated from the library, potentially representing mixed populations. Haemagglutination assays were performed with cell culture supernatant derived from infected AX4 cells; [b]Recreated viruses. Changes were engineered into the background of VN1203/PR8. Haemagglutination assays were carried out with concentrated viruses; [c]Viruses were tested for agglutination of untreated turkey red blood cells (TRBCs) possessing both Siaα2,3Gal and Siaα2,6Gal, or TRBC treated with a Siaα2,3-linkage-specific sialidase, leaving predominantly Siaα2,6Gal (i.e., human-type receptors). nd, not done.

Supplementary Table 2: Virus titres in tissues of ferrets infected with H5 avian-human reassortant viruses

Virus	Days after infection	Virus titre (mean $\log_{10}$(p.f.u. g^{-1}))						
		Nasal turbinates	Trachea	Lungs	Brain	Colon	Kidney	Spleen
rgCA04	3	8.0±0.3	6.4±1.0	4.2±1.9	-	1.9	-	-
	6	4.9±0.5	3.1	-	-	-	-	-
rgVN1203/CA04	3	7.3±0.8	5.0±0.3	2.3	-	2.1±0.6	-	-
	6	5.4±0.5	3.7, 3.9	-	-	3.0	-	-
rg(E119G/V152I/	3	2.4, 3.5	3.1	4.5	-	-	-	-
N224K/Q226L)/CA04	6	6.2, 3.3	-	-	-	-	-	-
rg(N224K/Q226L)/	3	5.5±2.5	2.4, 2.3	2.3, 4.3	-	-	-	-
CA04	6	6.1±1.8	-	4.8	-	1.7	-	-
HA(N158D/N224K/	3	8.7±0.2	5.4	3.8±0.7	2.3, 1.9	-	-	-
Q226L)/CA04	6	7.2±1.7	6.1	6.4, 5.2	-	2.8	-	-
HA(N158D/N224K/	3	8.7±0.2	3.5	3.6, 3.4	2.9	-	-	-
Q226L/T318I)/CA04	6	5.9±1.4	3.6, 3.4	4.2, 4.5	-	-	-	-
rgT318I/CA04	3	7.8±0.3	4.8±0.8	2.8	3.8, 3.0	-	2.2	2.1
	6	5.8±1.1	2.7, 3.3	4.2, 4.5	-	-	-	-

Ferrets were intranasally infected with 10^6 p.f.u. (500 μl) of virus. Three ferrets per group were euthanized on days 3 and 6 after infection for virus titration. When virus was not recovered from all three ferrets, individual titres were recorded. No virus was detected in liver samples. A dash indicates no virus isolation. Standard deviation is shown for virus titre values.

Supplementary Table 3: Amino acid changes in HA during replication of rg(N224K/Q226L)/CA04 virus in ferrets

Virus or virus samples:	Amino acid position		
	158	224	226
rgVN1203/CA04 virus	N	N	Q
rg(N224K/Q226L)/CA04 virus	N	K	L
Nasal turbinate/Ferret 1	N	K	L
Nasal turbinate/Ferret 2	D	K	L
Nasal turbinate/Ferret 3	K	K	L

Full genome sequences of viruses in the nasal turbinates of ferrets were determined. The nasal turbinates were collected on day 6 after infection. No mutations in the PB2, PB1, PA, NP, NA, M, or NS genes were detected.

Supplementary Table 4: Amino acid changes in HA during transmission of the HA(N158D/N224K/Q226L)/CA04 virus in ferrets

		Amino acid position					
		158	193	224	226	242	318
VN1203/CA04 virus		N	K	N	Q	A	T
HA(N158D/N224K/Q226L)/ CA04 virus		D	K	K	L	A	T
Pair 1	inoculated ferret	D	K	K	L	S	T
	contact ferret	D	N	K	L	S	T
Pair 2	inoculated ferret	D	K	K	L	A	I
	contact ferret	D	K	K	L	A	I
Pair 3	inoculated ferret	D	K	K	L	S	T
Pair 4	inoculated ferret	D	K	K	L	S	T
Pair 5	inoculated ferret	D	K	K	L	A/S[a]	T
Pair 6	inoculated ferret	D	K	K	L	S	T

Full genome sequences of viruses in the nasal washes from ferrets were determined. The nasal washes of infected ferrets and contact ferrets were collected on day 5 post-infection and on day 7 post-contact, respectively. No mutations in the PB2, PB1, PA, NP, NA, M, or NS genes were detected.

[a]Both A and S were detected.

Supplementary Table 5: Amino acid changes in HA during transmission of the HA(N158D/N224K/Q226L/T318I)/CA04 virus in ferrets

		Amino acid position					
		158	224	225	226	242	318
VN1203/CA04 virus		N	N	G	Q	A	T
HA(N158D/N224K/Q226L/T318I)/ CA04 virus		D	K	G	L	A	I
Pair 1	inoculated ferret	D	K	G	L	A/T[a]	I
	contact ferret[b]	D	K	G	L	A	I
Pair 2	inoculated ferret	D	N/K[a]	G/E[a]	L	A/T[a]	I
	contact ferret	D	K	G	L	T	I
Pair 3	inoculated ferret	D	N/K[a]	G	L	A/T[a]	I
	contact ferret[c]	D	K	G	L	T	I
Pair 4	inoculated ferret	D	K	G/E[a]	L	A/T[a]	I
Pair 5	inoculated ferret	D	K	G/E[a]	L	A/T[a]	I
	contact ferret	D	K	G/E[a]	L	A/T[a]	I
Pair 6	inoculated ferret	D	K	G	L	T	I

Full genome sequences of viruses in the nasal washes from ferrets were determined. The nasal washes of infected ferrets and contact ferrets were collected on day 5 post-infection and on day 7 post-contact, respectively. Virus was not isolated from the contact animals of pairs 4 and 6.

[a]Both residues were detected.

[b]A threonine to isoleucine change at position 430 in NP was detected.

[c]An asparagine to threonine change at position 4 in NA was detected.

Supplementary Table 6: Haemagglutination inhibition (HI) reactions of H5 HA reassortant viruses against post-vaccination sera

Virus	Human serum*	
	High titer pool (NR-4109)	Low titer pool (NR-4110)
VN1203/PR8	40†	20
N158D/N224K/Q226L/T318I	160	40

*NR-4109 and NR-4110 are a high titre pool and a low titre pool of polyclonal antiserum, respectively, from individuals vaccinated with a monovalent influenza subvirion vaccine, rgA/Vietnam/1203/2004×A/PR/8/34 (H5N1). These reagents were obtained from BEI Resources.

†Values are reciprocals of the highest serum dilutions that inhibit haemagglutination completely.

Supplementary Table 7: Virus susceptibility to oseltamivir

Virus	IC_{50} of oseltamivir carboxylate* (nM)
A/California/04/2009 (H1N1) (oseltamivir-susceptible control)	1.4 ± 0.3†
A/Osaka/180/2009 (H1N1) (oseltamivir-resistant control)	1133.5 ± 14.9
HA(N158D/N224K/Q226L/T318I)/CA04	1.0 ± 0.3

*Oseltamivir carboxylate is the active form of oseltamivir.

†IC_{50} value: mean ± standard deviation from triplicate assays of oseltamivir concentration needed to inhibit neuraminidase activity by 50%.

4. Supplementary references

1 Itoh, Y. *et al.* In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. *Nature* **460**, 1021-1025 (2009).

2 Stevens, J. *et al.* Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. *Science* **312**, 404-410 (2006).

3 Ha, Y., Stevens, D. J., Skehel, J. J. & Wiley, D. C. X-ray structures of H5 avian and H9 swine influenza virus hemagglutinins bound to avian and human receptor analogs. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 11181-11186 (2001).