# Supporting Information

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### SI Material and Methods

**Dataset Design and Phylogenetic Analysis.** Nucleotide sequences of the HA and NA genes of HPAI viruses were downloaded and aligned using the National Center for Biotechnology Information Influenza Virus Resource (1). Datasets and phylogeny were similar and in agreement with the WHO clade system datasets, but additional sequences were included in the alignment used for this study (2, 3). A resolved phylogeny required for ancestral sequence reconstruction was generated using PAUP v4b10 (4). Neighbor-joining tree search methods were optimized using an appropriate DNA substitution model, and γ-rate heterogeneity was determined with MrModeltest v2.2 (5). Estimates of statistical support for the observed phylogenies were calculated by performing 1,000 neighbor-joining bootstrap replicates.

**Viruses.** This study used H5N1 influenza A viruses VN1203 (clade 1), HK213 (clade 1), DKHUN795 (clade 2.1), WSM244 (clade 2.2), TYEGY7, (clade 2.2; accession numbers CY055188 to CY055195), JWEHK1038 (clade 2.3.4), DKLAO3295 (clade 2.3.4), and GG337 (clade 4). The viruses were propagated in 10-d-old embryonated chicken eggs and handled at St. Jude Children's Research Hospital in BSL-3+ facilities approved by the US Department of Agriculture. The four ancestral HA (A, B, C, and D) and the two ancestral NA (d and r) constructs were cloned into a dual-promoter plasmid, pHW2000. The rg viruses with ancestral HA and NA and the six internal genes from PR8 were generated by DNA transfection as described previously (6). Each viral HA and NA segment was sequenced to confirm the identity of the virus. The 6+2 rg viruses in PR8 backbone were also generated for VN1203 (7), HK213 (6), DKHUN795, WSM244, JWEHK1038, DKLAO3295, and GG337. For in vitro use, HPAI H5N1 HA cleavage sites were modified to match low-pathogenicity viruses so that the rg strains could be used in a BSL-2+ laboratory after exemption from US Select Agent classification. The 6+2 rg DKHUN795 in VN1203 backbone was also rescued in a BSL-3+ laboratory to serve as a lethal challenge virus in the ferret model.

**HI and MN Assays.** HI and MN assays were performed as described in the WHO manual on animal influenza diagnosis and surveillance (8). Briefly, ferret sera were treated with receptor-destroying enzyme (Denka Seiken) overnight at 37 °C, heat-inactivated at 56 °C for 30 min, diluted 1:10 with PBS solution, and tested by an HI assay with 0.5% packed chicken red blood cells. Filtered sera were used for MN assay. Neutralizing titers were expressed as the reciprocal of the serum dilution that inhibited 50% of viral growth of 100 50% tissue culture infectious dose of virus.

**ELISA.** The 96-well Microtest polystyrene assay plates (BD Bioscience) were coated overnight at 4 °C with 25 to 100 ng per well of the following H5N1 HA proteins: A/Vietnam/1203/04 (aa 18–530), A/Indonesia/5/05 (aa 17–530), A/Egypt/2321-NAMRU3/07 (aa 17–530), A/goose/Guiyang/337/06 (aa 17–530), and A/Japanese white-eye/Hong Kong/1038/06 [aa 17–530 (Eenzyme); mainly trimer/oligomer viral proteins, purified from 293 cell culture]. Blocking was performed for 2 h at 4 °C with 10% FCS. Ferret serum (50 μL/well; dilutions of 1:10–1:1,280) was added in duplicate and incubated for 2 h at 37 °C. Alkaline phosphatase-conjugated affinity-purified goat anti-ferret IgG γ (100 μL, 1:1,000; Rockland) was added to each well and incubated at 37 °C for 1 h. P-nitrophenylphosphate substrate (KPL) was finally added before the plates were read at 405 nm in a Model 680 microplate reader (Bio-Rad). Cutoff for positivity was set up for each dilution as three times the mean optical density of the negative sera. IgG titers were expressed as the reciprocal of the lowest positive serum dilution.

**Vaccine Preparation.** Vaccine strains rg 6+2 VN1203, DKHUN795, A, and D were propagated in the allantoic cavity of 10-d-old embryonated chicken eggs at 37 °C for 48 h. They were inactivated by addition of 1:2,000 β-propiolactone for at least 72 h at 4 °C. Inactivation was confirmed by the absence of virus growth after two passages in embryonated chicken eggs. Vaccines were concentrated by Amicon ultrafiltration and by passage through 25% and 70% sucrose cushion gradients. They were purified by ultracentrifugation through a continuous sucrose gradient for 2.5 h before they were pelleted by centrifugation at 76,000 × *g* for 1 h at 4 °C. The total protein content of the vaccines was measured with a protein assay (Bio-Rad) based on the Bradford method (9). Two-dimensional gel electrophoresis spot quantification (with MS identification of the picked spots) allowed estimation that the HA corresponded to approximately 25% of the total protein content in our H5 vaccines.

**Production of Ferret Antisera.** All animal experiments were approved by the Animal Care and Use Committee of St. Jude Children's Research Hospital and were performed in compliance with relevant institutional policies of the National Institutes of Health and the Animal Welfare Act. Before any experiment, ferrets were screened by HI assay to ensure seronegativity to the seasonal influenza viruses A/Brisbane/59/07 (H1N1) and A/Brisbane/10/07 (H3N2). Antisera against the four attenuated ancestor viruses A+r, B+r, C+r, and D+r, as well as against rg VN1203, HK213, DKHUN795, WSM244, JWEHK1038, DKLAO3295, and GG337, were produced in 3- to 4-mo-old ferrets from Triple F Farms. The animals were infected intranasally with $10^6$ $EID_{50}$ of 6+2 rg attenuated viruses, boosted 3 and 6 wk after the first infection with $10^7$ $EID_{50}$ of the same strain, and finally bled out for serum production.

**Ferret Nasal Washes.** On days 3, 5, and 7 after virus inoculation, ferrets were anesthetized with ketamine (25 mg/kg; Hospira), and nasal washes were collected. BSA (Sigma) was added at a ratio of 1:20 vol/vol as a stabilizing agent. Virus was titrated in eggs, and the $\log_{10}$ $EID_{50}$ per milliliter was calculated by the method of Reed and Muench (10). The limit of virus detection was lower than 0.75 $\log_{10}$ $EID_{50}$/mL virus titers, compared by ANOVA. For computation purposes, virus titers below the lower limit of detection were assigned a value of 0 [$\log_{10}(1)$].


1. Bao Y, et al. (2008) The influenza virus resource at the National Center for Biotechnology Information. *J Virol* 82:596–601.
2. World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization H5N1 Evolution Working Group. (2008) Toward a unified nomenclature system for highly pathogenic avian influenza virus (H5N1). *Emerg Infect Dis* 14:e1.
3. World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization H5N1 Evolution Working Group (2009) Continuing progress towards a unified nomenclature for the highly pathogenic H5N1 avian influenza viruses: Divergence of clade 2.2 viruses. *Influenza Other Respir Virus* 3:59–62.
4. Swofford DL (2001) *PAUP: Phylogenetic Analysis Using Parsimony (and Other Methods) 4.0 Beta* (Sinauer, Sunderland, MA).
5. Nylander JAA (2004) *MrModelTest, Version 2* (Uppsala Univ, Uppsala).
6. Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc Natl Acad Sci USA* 97:6108–6113.

7. Salomon R, et al. (2006) The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. *J Exp Med* 203:689–697.
8. World Health Organization (2002) *WHO Manual on Animal Diagnosis and Surveillance*. Available at http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf. Accessed August 8, 2010.
9. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254.
10. Reed LJ, Muench H (1938) A simple method for estimating fifty percent endpoints. *Am J Hygiene* 27:493–497.

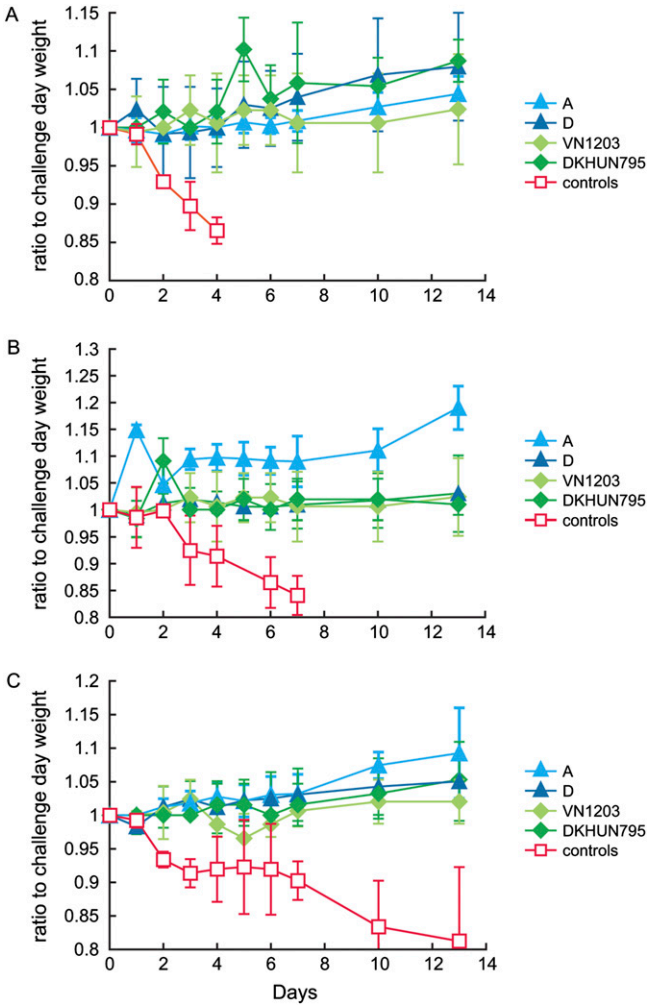


**Fig. S1.** Ferrets' weight loss after infection with VN1203 (*A*), DKHUN795 (*B*), and TYEGY7 (*C*). The mean weight loss as a ratio to challenge day weight are represented ± SEM. Control (mock-vaccinated) ferrets are represented by red open squares. Ferrets vaccinated with ancestral strains are represented by closed blue triangles: A in light blue and D in dark blue. Ferrets vaccinated with positive control isolate-based strains are represented by green closed diamonds: VN1203 in light green and DKHUN795 in dark green.

**Table S1. Growth properties of ancestral H5N1 viruses and 6+2 rg representatives of each H5N1 clade virus used in the present study**

| Virus | End point of $EID_{50}$/mL, 10^ | End point of $TCID_{50}$/mL, 10^ |
|---|---|---|
| A+r | 9.5 | 7.2 |
| B+r | 10.2 | 6.7 |
| C+r | 8.8 | 7.4 |
| D+r | 9.5 | 7.2 |
| A+d | 7.5 | 5.3 |
| B+d | 7.5 | 6.9 |
| C+d | 8.5 | 8.1 |
| D+d | 7.5 | 6.2 |
| VN1203 (clade 1) | 9.5 | 8.3 |
| DKHUN795 (clade 2.1) | 9.5 | 8.5 |
| WSM244 (clade 2.2) | 7.8 | 8.2 |
| GG337 (clade 4) | 8.5 | 7.7 |
| DKLAO3295 (clade 2.3.4) | 8.8 | 6.7 |

$TCID_{50}$, 50% tissue culture infectious dose.

**Table S2. HA aa mutations between A, D, VN1203 and DKHUN795 (HA ORF aa numbering)**

| Virus | 8 | 52 | 100 | 102 | 110 | 140 | 145 | 172 | 205 | 228 | 243 | 279 | 336 | 494 | 495 | 500 | 511 | 523 | 525 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | L | T | S | A | D | N | S | A | K | K | E | A | T | V | K | D | N | M | T |
| D | — | — | N | — | N | D | — | — | R | — | D | — | S | — | R | — | K | I | — |
| VN1203 | F | K | N | V | — | S | L | T | — | R | — | T | S | — | R | D | K | I | I |
| DKHUN 795 | — | — | N | — | N | D | — | — | R | — | — | — | S | I | R | N | K | I | — |

All aa identical to those of ancestral strain A aa are indicated by a dash.

**Table S3. Serum HI antibody titers before and after challenge in ferrets vaccinated with inactivated whole-virus H5N1 influenza vaccines**

| Vaccination regimen/challenge virus | Prechallenge arithmetic HI mean titer (against vaccine/challenge virus) with chicken red blood cells* | Mean HI titer against vaccine (homologous) with chicken red blood cells* |
|---|---|---|
| rg A | | |
| WT VN1203 | 42.5/23.75 | 37 |
| rg DKHUN795† | 21.25/5 | |
| WT TYEGY7 | 46.25/22.5 | |
| rg D | | |
| WT VN1203 | 91.25/5 | 95 |
| rg DKHUN795† | 85/55 | |
| WT TYEGY7 | 107.5/32.5 | |
| rg VN1203 | | |
| WT VN1203 | 31.25/31.25‡ | 26 |
| rg DKHUN795† | 33.75/28.75 | |
| WT TYEGY7 | 13.75/5 | |
| rg DKHUN795 | | |
| WT VN1203 | 65/5 | 87 |
| rg DKHUN795† | 55/55‡ | |
| WT TYEGY7 | 140/57.5 | |
| PBS | | |
| WT VN1203 | —/5 | — |
| rg DKHUN795† | —/5 | |
| WT TYEGY7 | —/5 | |

*rg WSM244 was used instead of TYEGY7. Both viruses belong to clade 2.2.
†rg 6+2 DKHUN795 in VN1203 backbone.
‡Homologous challenge: same vaccine and challenge strains.

Attenuated rg 6+2 viruses (in PR8 backbone and with a low pathogenic HA cleavage site) were used so that the assays could be performed in a BSL2+ laboratory. rg, reverse genetics 6+2 in A/Puerto Rico/8/34 backbone unless specifically indicated.

## Other Supporting Information Files

Appendix S1 (DOC)