Supporting Information

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SI Materials and Methods

Virion Protein Quantification. Viruses were purified by ultracentrifugation on a stepped 15–60% sucrose gradient. Virus was collected from the interface of 15% and 60% sucrose, pelleted, and reconstituted in PBS. Serial dilutions of virus then were boiled in SDS containing DTT (100 mM), resolved by polyacrylamide electrophoresis, and transferred to PVDF membranes. Blots were probed with mouse anti-HA mAb RA5-22, mouse anti-M1 mAb M2-1C6, and rabbit anti-NA C-terminal polyclonal antibody (pAb), followed by incubation with anti-mouse and anti-rabbit secondary mAbs conjugated with 680-nm and 800-nm IRDye (LI-COR). Blots then were visualized simultaneously, and protein was quantitated on an Odyssey infrared scanner using Image Studio v2.0 software (LI-COR).

For ELISA, half-area 96-well high-binding ELISA plates (Greiner Bio-One) were incubated with serial dilutions of nasal wash samples overnight at 4 °C. Plates were blocked with 0.1% BSA in PBS for 2 h at room temperature, washed with PBS + 0.05% Tween-20, and incubated for 3 h at room temperature with a mixture of four HA-specific mouse mAbs recognizing distinct epitopes. After extensive washing with PBS + 0.05% Tween-20, bound primary antibodies were detected by incubation with an HRP-conjugated rat anti-mouse IgG kappa light chain antibody for 2 h at room temperature. ELISA plates were washed again with PBS + 0.05% Tween-20, developed with the LumiGlo chemiluminescent substrate (Kirkegaard & Perry Lab, Inc.), and read on a SpectroMax M5 luminometer (Molecular Devices). Signals for dilutions in the linear range were used to calculate the ratio of HA protein to NA activity for each sample.

NA Activity Assay. Neuraminidase activity was determined for the same purified particle preparations used for the assessment of total protein content. Activity was determined using a fluorogenic substrate, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (Sigma), as previously described (1). Briefly, virus was serially diluted twofold in assay buffer [33 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5), 4 mM $CaCl_2$]. Substrate was added to diluted virus samples at a final concentration of 200 μM. Reactions were incubated at 37 °C, and fluorescence was measured every minute over a 30-min period on a Spectromax M5 microplate reader (Molecular Devices). Michaelis–Menten kinetics were determined for each dilution of virus to obtain the V_{max} value for comparison.

RNA Quantification. The vRNA content in purified virus preparations was determined by quantitative RT-PCR. RNA was extracted using the QiaAmp viral RNA extraction Kit (Qiagen) and was "reverse transcribed" with the Verso RT kit (Thermo Scientific) using a universal primer specific for the 3′ end of the vRNA 5′-AGCAAAAGCAGG-3′. Quantitative PCR on cDNA was carried out using the Power SYBR Green PCR Master Mix (Invitrogen/Life Technologies) on a Mastercycler ep Realplex thermal cycler (Eppendorf). PCR primer binding sites were within the first 150 bp of the 3′ end (HA and NA segments) or within 170 bp of the 5′ end (M segment). PCR primers used: HA: 3′-AAGGCAAACCTACTGGTCCTGTT-5′, 3′-AATTGTTCGCATGGTAGCCTATAC-5′; NA: 3′-AAATCAGAAAATAACAACCATTGGA-5′, 3′-ATTCCCTATTTGCAATATTAGGCT-5′; M: 3′-ACAGAGACTTGAAGATGTC-5′, 3′-TCTTTAGCCATTCCATGAG-5′.

To generate singly infected cells for RNA analysis, MDCK cells were infected with $PR8_{WT}$ or $PR8_{NP:F346S}$ in triplicate at an MOI of 0.2. At 7 hpi, cells were harvested and surface stained with Alexa Fluor 488-conjugated mouse anti-HA mAb H36-26 and Alexa Fluor 647-conjugated mouse anti-NA mAb NA2-1C1. Approximately 10^6 HA^+NA^+ cells then were sorted from each replicate using a FACS Aria sorter (BD Biosciences), and RNA was extracted using the RNeasy mini kit (Qiagen).

Strand-specific RT-PCR then was used to measure the relative abundance of viral vRNA and mRNA in cells, as described elsewhere, but with primers specific for PR8 (2). Briefly, hot-start reverse transcription [Superscript III (Invitrogen) in trehalose-supplemented buffer] using tagged RT primers targeting the 5′ end of the vRNA and the 3′ end of the mRNA was followed by quantitative real-time PCR as described above using segment-specific internal primers in combination with primers specific for the tag on the mRNA or vRNA cDNA primers. This method distinguishes between vRNA, mRNA, and cRNA of a particular gene segment.

Analysis of Single Virion Expression. MDCK cells were infected with virus diluted to ensure that <5% of cells were infected. For $PR8_{WT}$ and $PR8_{NP:F346S}$, neutralizing anti-HA mouse mAb H17-L2 (5 μg/mL) was added to cultures at 2 hpi to prevent spread. For other H1N1 strains, spread was blocked by changing to minimal essential medium with 50 mM Hepes, 20 mM NH_4Cl, pH 7.2, at 3 hpi. At 16 hpi, cells simultaneously were fixed and permeabilized in foxP3 fix/perm buffer (eBioscience). $PR8_{WT}$- and $PR8_{NP:F346S}$-infected cells were stained with Alexa Fluor 488-conjugated mouse anti-HA mAb H36-26 (which does not compete with H17-L2), Pacific Orange-conjugated mouse anti-NA mAb NA2-1C1, Alexa Fluor 647-conjugated mouse anti-NP mAb HB-65, and Pacific Blue-conjugated mouse anti-NS1 mAb NS1-1A7. Other H1N1 strains used were stained with human anti-HA mAb 2G02 (generously provided by Patrick C. Wilson, University of Chicago, Chicago) followed by Alex Fluor 488-conjugated donkey anti-human secondary (Jackson ImmunoResearch), rabbit anti-NA C′ tail pAb followed by PE-conjugated donkey anti-rabbit secondary (also from Jackson ImmunoResearch), and Alexa Fluor 647-conjugated mouse anti-NP mAb HB-65. After staining, cells were washed, run on a BD LSR II, and analyzed using FlowJo version 9.3 (Tree Star, Inc.). Population expression frequencies were calculated by looking at all infected cells, as determined by positive staining against at least one of the viral proteins examined, and then determining the percentage of infected cells expressing each examined gene product.

Quantification of Single-Round Virus Output. MDCK cells in six-well plates were infected with $PR8_{WT}$ or $PR8_{NP:F346S}$ at an MOI of 0.01 $TCID_{50}$ per cell in the presence of 1 μg/mL TPCK-treated trypsin. At 8–9 hpi, supernatants were collected, clarified, and aliquoted. At the time of collection, producer cell monolayers were disrupted into single-cell suspensions, and live cells were enumerated by Trypan Blue exclusion. Producer cells then were fixed and permeabilized in foxP3 fix/perm buffer (eBioscience), and the frequencies of $HA^+NP^+NA^+NS1^+$ cells were determined by flow cytometry as described above. These frequencies were multiplied by the total cell counts to estimate the number of productively infected cells present in each monolayer. Supernatants then were quantified for $TCID_{50}$ and SI particle concentrations, using the methods described above, with SI particles measured based on the ability to express HA or NP. The data presented are pooled from two independent experiments using

independently rescued stocks of the two viruses. Each data point represents the value from a single well of a six-well plate.

Virus-Free Expression Analysis. HeLa cells in a 24-well plate were transfected with plasmids expressing PB2, PB1, PA, and NP (wild-type or NP:F346S) from the PR8 eight-plasmid reverse genetics rescue system described in *Materials and Methods* (0.125 μg per well each), along with plasmids expressing PR8 HA and NA vRNA from a human RNA polymerase I promoter (pHH21 vector generously provided by Andrew Pekosz, Johns Hopkins School of Public Health, Baltimore; 0.25 μg per well each). Transfections were carried out using Lipofectamine 2000 (Life Technologies). Twenty-four hours after transfection, cells were simultaneously fixed and permeabilized in foxP3 fix/perm buffer (eBioscience) and were stained with Alexa Fluor 488-conjugated mouse anti-HA mAb H36-26, Pacific Orange-conjugated mouse anti-NA mAb NA2-1C1, and Alexa Fluor 647-conjugated mouse anti-NP mAb HB-65. After staining, cells were washed, run on a BD LSR II flow cytometer system, and analyzed using FlowJo version 9.3 (Tree Star, Inc.). HA and NA geometric mean fluorescence intensities (GMFI) were determined for the HA^+ and NA^+ cell populations, respectively.

Evaluation of NA Gene Dose on Viral Protein Expression. Vero cells were infected in triplicate with $PR8_{WT}$ at an MOI of 10 HAEU per cell plus 0, 10, 20, 40, or 80 HAEU per cell of $PR8_{noNA}$, and at 2 hpi neutralizing anti-HA mouse mAb H17-L2 (5 μg/mL) was added to prevent spread. At 15 hpi, cells were harvested, fixed, and stained as described for single-virion analysis. Within the HA^+NA^+ population, we determined per-cell fluorescence ratios for HA, NP, NA, and NS1 using FlowJo version 9.3 (Tree Star, Inc.).

Guinea Pig Infections. Eight-week old SPF female Hartley guinea pigs (Charles River) were inoculated while under isoflurane anesthesia using 300 μL of virus diluted in balanced salt solution supplemented with 0.1% BSA. Virus was applied dropwise into the nares. Nasal washes were performed on isoflurane-anesthetized animals by dispensing 1 mL of PBS supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin into one nostril, and collecting flow-through from the other nostril. Nasal washes were frozen immediately on dry ice and subsequently were quantitated for infectious virus by $TCID_{50}$ assay on MDCK cells.

Analysis of Guinea Pig Nasal Turbinate Cells. At 9 or 48 hpi, guinea pigs were killed by intracardiac pentobarbital injection according to NIAID IACUC guidelines. Nasal turbinates were collected into RPMI + 25 mM Hepes, 7.4% FBS, 800 U/mL type I collagenase (Worthington), and 25 U/mL DNase (also Worthington). Tissues were disrupted further with bone cutters and then were digested at 37 °C with shaking for 1 h. Suspensions were passed through a 70-μm nylon screen and were spun over a lymphocyte separation medium cushion (Lonza). The interface was collected and treated with ammonium-chloride-potassium buffer (Lonza) to remove remaining RBCs. After washing, cells were counted and fixed/permeabilized using foxP3 fix/perm buffer (eBioscience). Cells then were stained with Alexa Fluor 488-conjugated mouse anti-HA mAb H36-26, Alexa Fluor 647-conjugated mouse anti-NA mAb NA2-1C1, and Pacific Blue-conjugated mouse anti-NP mAb HB-65, run on a BD LSR II flow cytometer system, and analyzed using FlowJo version 9.3 (Tree Star, Inc.). Live cells were gated based on the forward scatter A (FSC-A), side-scatter A (SSC-A), and SSC-W profile, followed by the exclusion of autofluorescent cells for some experiments. For viral protein-expression analysis, the per-cell fluorescence ratios of HA, NP, and NA were determined for the $HA^+NP^+NA^+$ population. To examine coexpression frequency, NP^+ cells were assessed for HA and NA expression using gates derived from full-minus-one staining controls.

1. Lambré CR, Chauvaux S, Pilatte Y (1989) Fluorometric assay for the measurement of viral neuraminidase in influenza vaccines. *Vaccine* 7(2):104–105.

2. Kawakami E, et al. (2011) Strand-specific real-time RT-PCR for distinguishing influenza vRNA, cRNA, and mRNA. *J Virol Methods* 173(1):1–6.

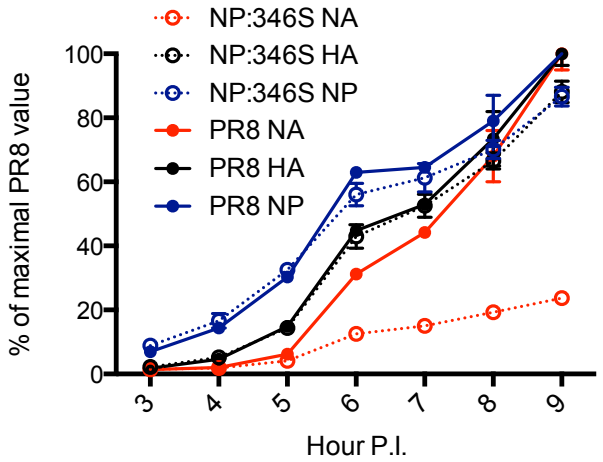

Fig. S1. NP:F346S decreases NA expression in cells infected at a high MOI. Intracellular HA, NP, and NA expression over time in MDCK cells infected with 20 HAEU of $PR8_{WT}$ or $PR8_{NP:F346S}$, as determined by flow cytometry and presented as the mean percent of mean $PR8_{WT}$ values at 9 h ± SEM for three technical replicates per time point.

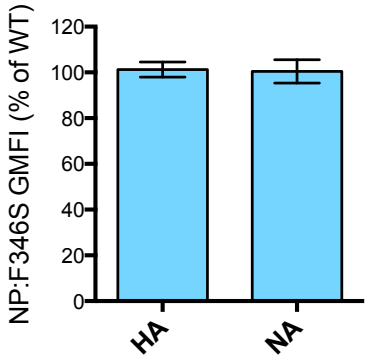

Fig. S2. NP:F346S does not reduce NA expression when NA vRNA is provided in excess. HA and NA expression in HeLa cells, as determined by flow cytometry, 24 h after transfection with expression plasmids encoding PB2, PB1, PA, and NP (wild-type or NP:F346S), along with plasmids that express the HA and NA vRNAs from a human RNA polymerase I promoter. Data are pooled from two independent experiments and represent the mean $PR8_{NP:F346S}$ values as a percentage of $PR8_{WT}$ ± SEM.

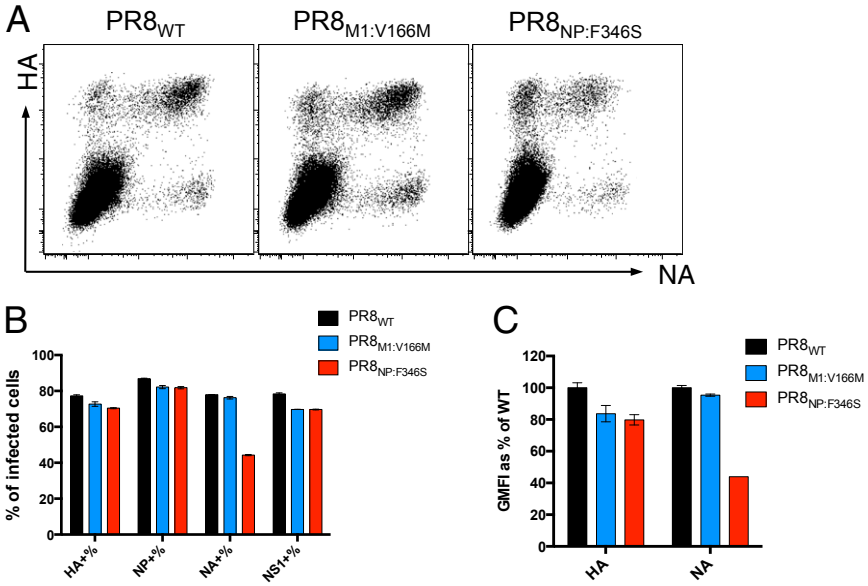

Fig. S3. M1:V166M does not affect the expression pattern of PR8. MDCK cells were infected with the indicated viruses at an MOI of 0.05 $TCID_{50}$. Anti-HA neutralizing Ab H17L2 (5 μg/mL) was added 1 hpi to block spread. At 16 hpi, cells were harvested, fixed, and permeabilized, stained against HA, NA, NP, and NS1, and run on an LSR II flow cytometer. (*A*) Representative dot plots showing HA and NA expression. (*B*) The population expression pattern illustrated as the percentage of cells in *A* that expressed detectable levels of the indicated proteins. Data represent the mean ± SEM of three technical replicates. (*C*) GMFI of stain against the indicated proteins within cells staining positive for that protein, presented as a percentage of $PR8_{WT}$ values. Data represent mean ± SEM.

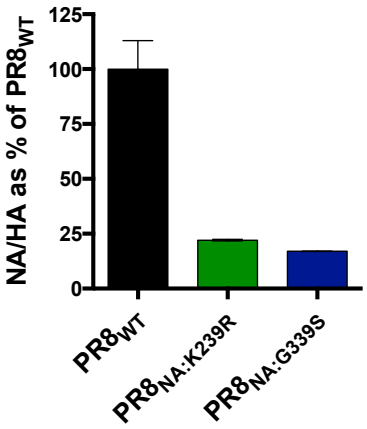

Fig. S4. The effect of NA mutations on the incorporation of NA protein into virions. The indicated viruses were purified using a sucrose gradient, and the relative amounts of HA and NA protein were determined by quantitative Western blot. Data represent the mean values for duplicate gel lanes ± SEM.

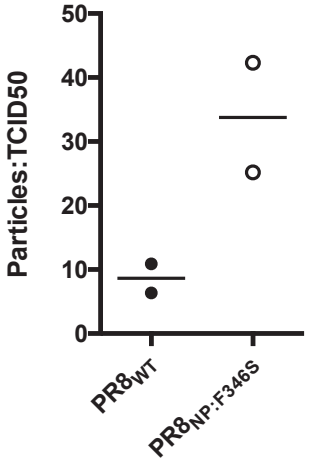

Fig. S5. NP:F346S increases the particle:$TCID_{50}$ ratio of PR8. The concentrations of physical particles (calculated by determining the furthest dilution of virus capable of agglutinating a predetermined number of turkey RBCs) and $TCID_{50}$ were determined for two independent reverse genetics-generated stocks of $PR8_{WT}$ and $PR8_{NP:F346S}$. Data represent the particle:$TCID_{50}$ ratios for individual stocks of virus.

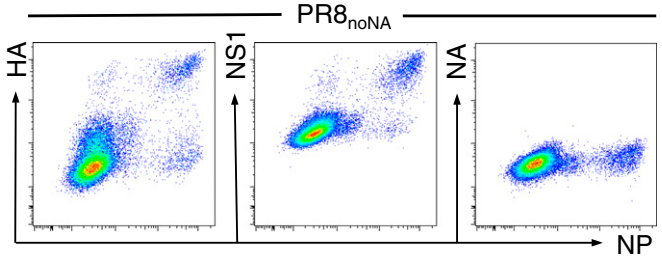

Fig. S6. Expression pattern of $PR8_{noNA}$. Representative dot plots showing HA, NP, NA, and NS1 expression in MDCK cells 16 h after low-MOI infection with $PR8_{noNA}$.

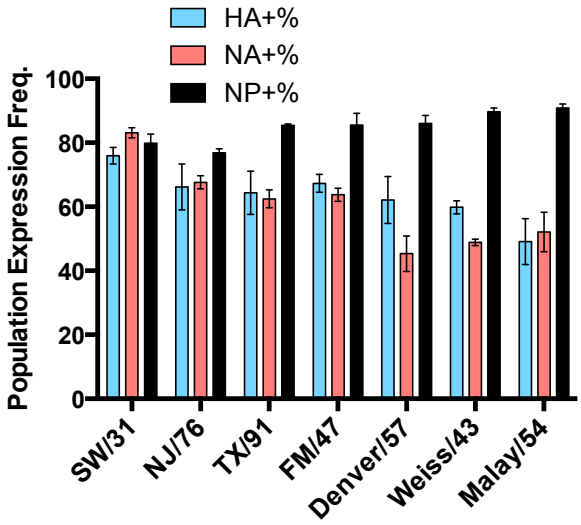

Fig. S7. Expression pattern of diverse H1N1 historical isolates. Population expression patterns in the indicated H1N1 strains as determined by flow cytometric analysis of MDCK cells infected at an MOI <0.05 $TCID_{50}$ per cell. Data represent mean ± SEM of values from three stocks of each strain.