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Supplementary Information for

H5N8 and H7N9 packaging signals constrain HA reassortment with a seasonal H3N2 influenza A virus

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Supplementary Information Text

Materials and Methods

Cells and virus. Madin-Darby canine kidney (MDCK) cells were a gift of Peter Palese (Icahn School of Medicine at Mount Sinai) and were grown using minimum essential medium (Gibco) supplemented with penicillin-streptomycin and 10% fetal bovine serum (FBS). Human embryonic kidney 293T cells were obtained from the American Type Culture Collection (ATCC) and were grown using Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% FBS. Reverse genetics-derived influenza A/Panama/2007/99 (H3N2; Pan/99) virus grown in 9- to 11-day old embryonated hens' eggs was used for all experiments.

Design of chimeric HA segments and virus generation. The full Pan/99 HA ORF was flanked with HA packaging signals (136 nt from the terminal 3' end and 136 nt from the terminal 5' end) originating from H5N8, H7N9, or H3N2 (Pan/99) viruses. In order to disrupt native packaging function, the first 20 and last 20 codons of the ORF with the exception of start, stop, met, and trp codons were changed to synonymous codons. ATG codons in the introduced 3' regions were changed to ATT to ensure proper translation of HA protein. BsmBI restriction sites were introduced to the ends of each construct to allow for ligation into the pDP2002 plasmid (Daniel Perez, UGA). HA constructs were then synthesized through Genewiz. The PSmut_HA construct did not contain introduced packaging signals; instead, it comprised only the standard length Pan/99 HA segment with the same mutations in the terminal 20 codons at either end of the HA ORF. Viruses were rescued by plasmid transfection of 293T cells and injection of 293T cell suspension into 9- to 11-day old hens' eggs (Hy-Line) after 17-24 h (1, 2). Following inoculation, eggs were incubated for 48 h at 33° C. Rescued viruses were then plaque-purified to minimize defective interfering particles and egg passage 1 stocks were generated by inoculating eggs with 100 PFU from a single plaque pick and incubating for 48 h at 33° C. All eight segments were genotyped by HRM to confirm WT or VAR identity and HA segment termini were Sanger sequenced (Genewiz) to ensure all modifications were maintained during virus rescue and propagation.

Virus growth in culture and in vivo. MDCK cells were inoculated at an MOI of 5 PFU/cell in triplicate with each virus. Virus attachment was performed on ice for 45 min, after which time the cells were washed 4x with cold PBS to remove residual inoculum. Warm media was then added to allow virus entry and the cells were incubated at 33° C. At 3 h p.i., ammonium chloride was added to the medium at a final concentration of 20 mM which, in conjunction with the absence of trypsin, ensures a single cycle of virus replication. Supernatants were sampled at 1, 4, 8, 12, 16, and 24 h p.i. and titered via plaque assay on MDCK cells. Three-week-old female Hartley strain guinea pigs weighing 300 to 350 g were acquired from Charles River Laboratories. Before intranasal inoculation, nasal lavage, or CO_2 euthanasia, the guinea pigs were sedated with an injection of ketamine plus xylazine (30 mg/kg and 4 mg/kg of body

weight, respectively). Twelve animals were inoculated with single virus diluted in 1x PBS: four animals received HA_H3PS virus, four animals received HA_H5PS virus, and four animals received HA_H7PS virus. After 16 h (day 1 p.i.), nasal lavage of all animals using 1x PBS was performed. Twenty four hours later, nasal lavage of all animals was again performed and repeated daily in this manner until the end of the experiment (day 5 p.i.). Nasal wash samples were thoroughly mixed post-acquisition and titered via plaque assay on MDCK cells.

Co-infection of MDCK cells and reassortment analysis. The control co-infections used WT and VAR viruses with homologous Pan/99 packaging signals on HA, and the heterologous co-infections used WT viruses with either H5N8 or H7N9 packaging signals on HA and a VAR virus with homologous Pan/99 packaging signals on HA as shown in Fig. 1 A-C**.** For the PSmut_HA co-infections, the WT virus contained the HA segment with mutated packaging signal regions while the VAR virus contained an HA segment with intact packaging signals as shown in *SI Appendix*, Fig. S2 A. MDCK cells were co-infected on ice and, once virus attachment had occurred, cells were washed with cold PBS and virus entry was synchronized by the addition of warm media in the absence of trypsin. Infection proceeded at 33° C, and the media was changed at 3 h p.i. to contain HEPES buffer and 20 mM ammonium chloride. The co-infection supernatants were harvested 12 h p.i. and plaque-purified on MDCK cells. Viral RNA was extracted from the plaque picks using the ZR-96 Viral RNA kit (Zymo Research), eluted in 40 µL water, and 12.8 µL RNA was used to make cDNA following the manufacturer's instructions (Maxima RT, Fermentas). qPCR utilizing Precision Melt Supermix (Bio-Rad) and IAV segment-specific primers was then performed in 384-well plates. The primers used were designed to bind to Pan/99 segments in a way that frames the VAR mutation site(s). qPCR was followed by a melt curve analysis, which allows for differentiation of WT vs VAR segments based on the presence or absence of the VAR mutations, which alter the melting properties of the DNA. The melt curve data were analyzed using Precision Melt Analysis software (Bio-Rad) and alignment to Pan/99 WT and VAR cDNA controls. The percentage of reassortant virus isolates that packaged a WT segment for each of the eight IAV segments was then calculated and graphed.

Droplet digital PCR (ddPCR). To measure replication over time of the various modified HA segments, MDCK cells were co-infected in triplicate at an MOI of 5 PFU/cell of each virus. After a 45 min attachment period on ice, cells were washed 3x with cold PBS and infection was initiated by addition of warm media in the absence of trypsin. Cells were incubated at 33° C for the duration of the experiment, and at 3 h p.i. media was supplemented with ammonium chloride at a final concentration of 20 mM. At 1, 4, 7, and 10 h p.i., co-infected cells and supernatants were harvested together as a single sample in lysis buffer + BME (Invitrogen), vortexed well to ensure thorough mixing, and frozen at -80° C. Once the time course had been completed, RNA was extracted from all samples using the PureLink RNA mini kit

(Invitrogen) following the manufacturer's instructions with the following modifications: the dry spin was performed for 2 min and 30 µL RNase-free water was used to elute the RNA after incubating on the column membranes for 2 min. Two micrograms of RNA from each sample was used for cDNA conversion (Maxima RT, Fermentas), and the cDNAs were subsequently diluted so that their concentrations were within the linear range of the ddPCR assay. PCR was set up using 8.8 µL diluted cDNA, 11 µL QX200 EvaGreen Supermix (Bio-Rad), and 2.2 µL primer (4 µM of F+R primer specific for the 3' HA packaging signal region of interest). These reactions were combined with 70 µL Droplet Generation Oil for EvaGreen (Bio-Rad), and a QX200 Droplet Generator (Bio-Rad) was used to generate droplets. Forty microliters of the generated droplets were then carefully transferred to a Twin-Tec 96-well PCR plate (Eppendorf) and PCR was carried out in a C1000 Touch Thermal Cycler (Bio-Rad) as follows: 95° C for 5 min, [95° C for 30 sec followed by 53.7° C for 1 min] x 40, 4° C for 5 min, 90° C for 5 min, and a final hold at 4° C. The ramp rate for the reaction steps was set at 2° C/sec as recommended by the manufacturer. After bringing the reactions to room temperature, the plate was read in a QX200 Droplet Reader (Bio-Rad) and the data were analyzed using QuantaSoft software (Bio-Rad), using the negative control values to define the background.

Co-infection and virus transmission in guinea pigs. Three-week-old female Hartley strain guinea pigs weighing 250 to 350 g were acquired from Charles River Laboratories. Before intranasal inoculation, nasal lavage, or CO_2 euthanasia, the guinea pigs were sedated with an injection of ketamine plus xylazine (30 mg/kg and 4 mg/kg of body weight, respectively). Eight animals were inoculated with WT:VAR virus mixtures: four animals received HA_H3PS plus HA_H5PS viruses and four animals received HA_H3PS plus HA_H7PS viruses. After 16 h (d 1 p.i.), nasal lavage using 1x PBS was performed on the eight inoculated animals. Post-nasal lavage, a naïve animal was placed with each inoculated animal, so that eight cages housed one inoculated and one contact animal. Cages were then placed in Caron 6040 environmental chambers fitted with the optional dryer package set to 10° C and 20% relative humidity. Two environmental chambers were used, one chamber for HA_H3PS plus HA_H5PS viruses and one chamber for HA_H3PS plus HA_H7PS viruses. Twenty four hours later, nasal lavage of all 16 animals was performed. Contact animals were sampled prior to the inoculated animals, and care was taken to avoid cross-contamination between groups. Nasal lavage was repeated in this manner every 24 h until the end of the experiment, which ended day 6 p.i. for the inoculated animals and day 7 p.i. for the contact animals.

Nasal wash samples, which were not centrifuged prior to downstream applications, were titered via plaque assay on MDCK cells and processed for reassortment analysis in the same manner as the cell culture supernatants. To evaluate transmission to contacts or HA segment composition in donor (inoculated) animals, RNA was extracted from 140 µL of each of the guinea pig nasal wash samples

using the QIAamp Viral RNA mini kit (QIAGEN) following the manufacturer's instructions with the following modifications: carrier RNA was not added to the lysis buffer, the dry spin was performed for 2 min, and 40 µL RNase-free water was used to elute the RNA after incubating on the column membranes for 2 min. Twelve microliters of the resulting viral RNA was converted into cDNA (Maxima RT, Fermentas), and ddPCR was performed with 3' HA packaging signal-specific primers to enumerate total HA copies in the nasal wash samples as described above.

Virus stock concentration and HA segment enumeration. Virus stocks were grown in eggs (100 PFU/egg) at 33° C for 2 days. Each virus stock was grown in three eggs which served as three technical replicates for the experiment. Allantoic fluid samples were spun at 3,000 rpm for 10 min to pellet debris, then further clarified using a Beckman Coulter Optima XL-100K ultracentrifuge (10,000 rpm for 30 min at 4° C). Clarified supernatants were then transferred to new tubes and a 1.5 mL 25% sucrose in NTE buffer cushion was added to the bottom of the tubes. Tubes were spun at 25,000 rpm for 2 h at 4° C to pellet the virus. Virus pellets were then gently resuspended in PBS and incubated at 4° C for 30 min – 2 h. Viral RNA was then extracted from the pellets using the QIAamp Viral RNA mini kit (QIAGEN) following the manufacturer's instructions with the following modifications: carrier RNA was not added to the lysis buffer, the dry spin was performed for 2 min, and 40 µL RNase-free water was used to elute the RNA after incubating on the column membranes for 2 min. Twelve microliters of this RNA was used for cDNA conversion (Maxima RT, Fermentas) and ddPCR was performed using IAV segment-specific primers. For the non-HA segments, terminal primers (within 150 nt of the 3' end of the vRNA) were used to ensure efficient detection of both standard-length segments and any segments carrying internal deletions. Due to differences in the packaging signal regions of the various HA segments, internal primers were used for detection of this segment to allow for primer consistency across virus samples. In this way, the same primers were used across all virus samples. To ensure robustness of HA segment enumeration, two independent sets of PB2 and HA primers were employed (one set in Fig. 5A and a second set in Fig. 5B).

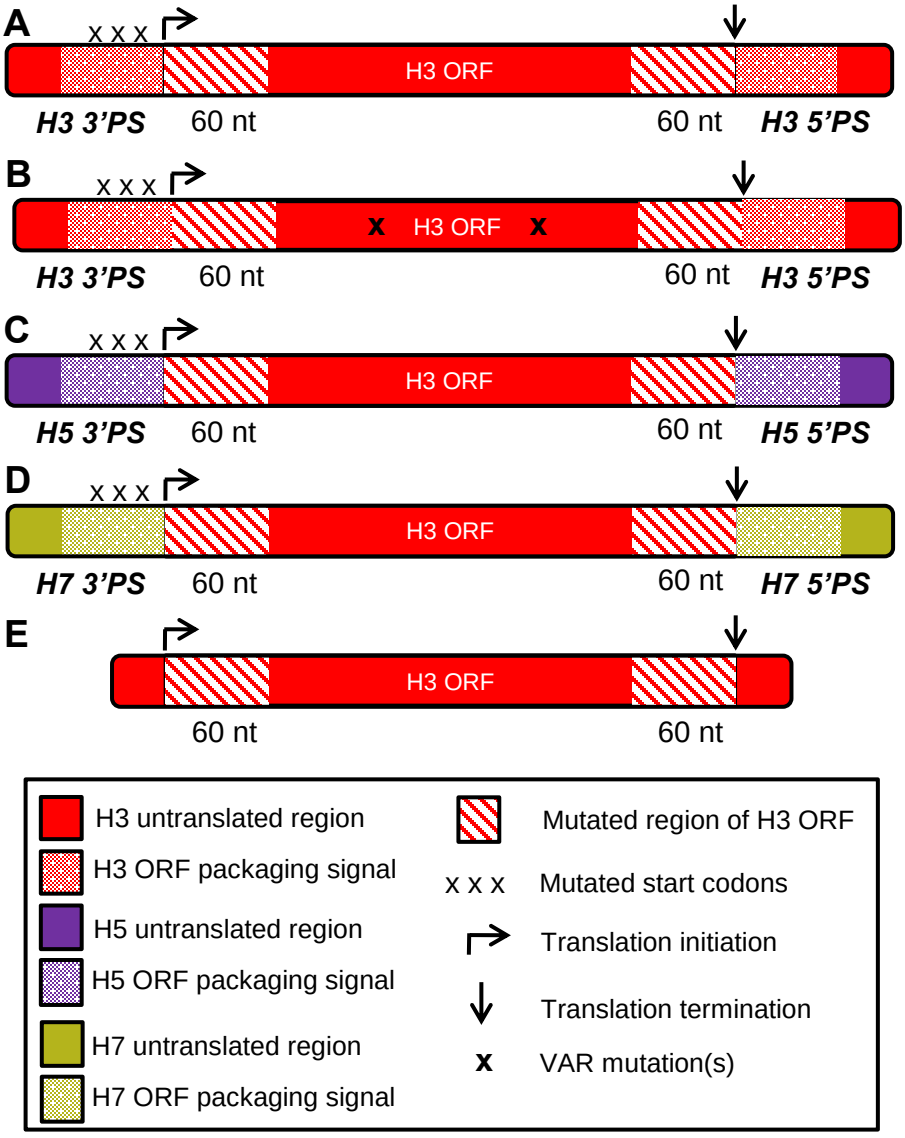

Fig. S1. Pan/99 HA segments constructed have identical ORFs but differing packaging signal regions. Chimeric HA segments were designed so that each segment encoded Pan/99 protein but carried packaging signals originating from H3N2 (Pan/99) (A and B), H5N8 (C), or H7N9 (D). The ORFs were silently mutated within 60 nt terminal regions (hatched boxes) so that packaging would be directed by the introduced 3' and 5' regions. The PSmut_HA segment (E) did not contain introduced packaging signals and was designed to test the disruption of packaging signal function in the ORF. Segments in (A, C, D, and E) are untagged (WT) while the segment in (B) contains silent mutations in the ORF that serve as genetic identifiers (VAR). Segments are shown in the positive sense and not to scale.

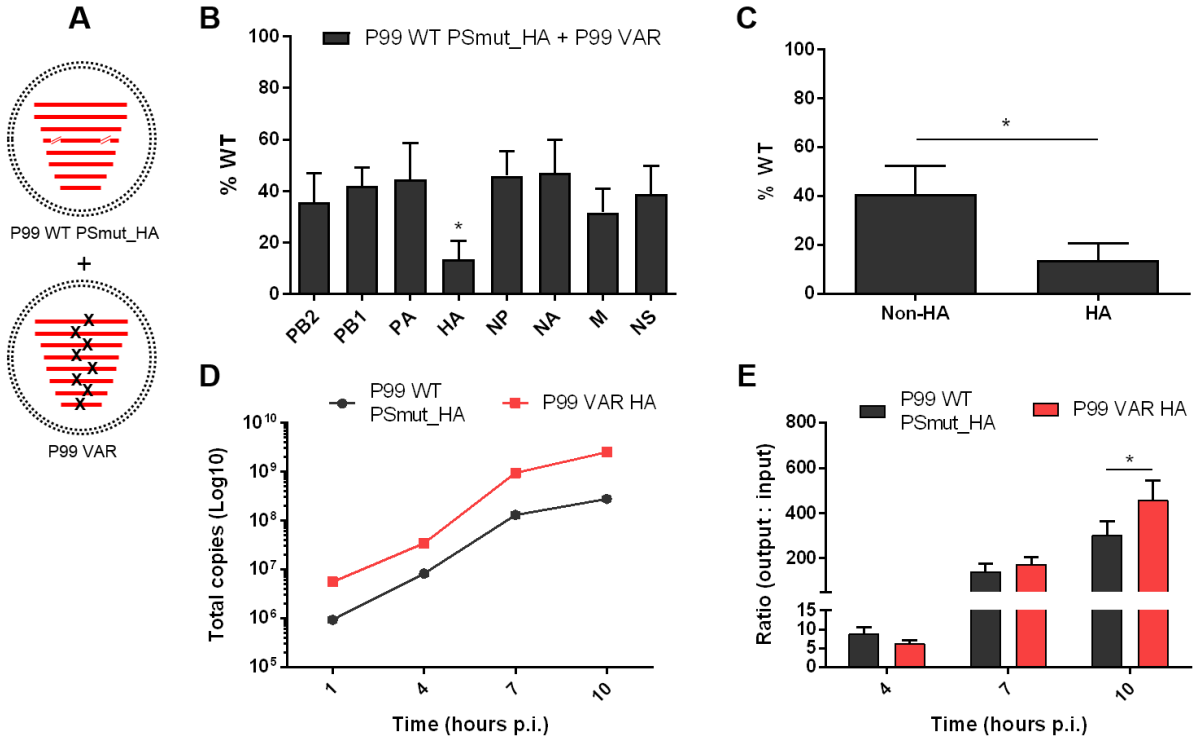

Fig. S2. The introduced HA ORF mutations disrupted packaging function but did not markedly affect segment replication efficiency. MDCK cells were co-infected at a high MOI with a WT virus carrying a full-length Pan/99 HA segment with silent mutations in the packaging signal regions of the ORF (red slash marks) and a VAR virus carrying a full-length Pan/99 HA segment with intact packaging signals in the ORF as shown in (A). Supernatants were harvested at 12 h p.i. and virus isolates were genotyped via HRM analysis. (B) The percentage of virus isolates that carried a WT segment for each of the eight IAV segments is plotted. Only reassortant virus isolates were included in the analysis. N = 8 (two biological replicates with four technical replicates each). Mean with SD is shown. (C) Statistical analysis of data presented in (B). Unpaired, two sided *t* test was used to analyze the difference between % WT for the PSmut_HA segment vs unmodified (non-HA) segments; *p < 0.0001. (D) To evaluate segment replication efficiency, MDCK cells were co-infected at a high MOI with the viruses shown in (A). At 1, 4, 7, and 10 h p.i., cells and supernatants were harvested as a single sample and RT ddPCR was performed to enumerate total copies of each HA segment over time. Primers used were specific for the 3' HA packaging signal region of each segment, with the forward primer binding in the region of the silent ORF mutations (or lack thereof). N = 3 (three technical replicates with ddPCR performed in duplicate for each). Mean with SD is shown. (E) Because results plotted in (D) suggested a difference in the input amount of the two viruses, HA segment replication over time was normalized by dividing the 4, 7, and 10 h output values by the 1 h input values and resulting ratios were graphed. N = 3; *p = 0.0041 using two-way ANOVA with Sidak's multiple comparisons. Mean with SD is shown.

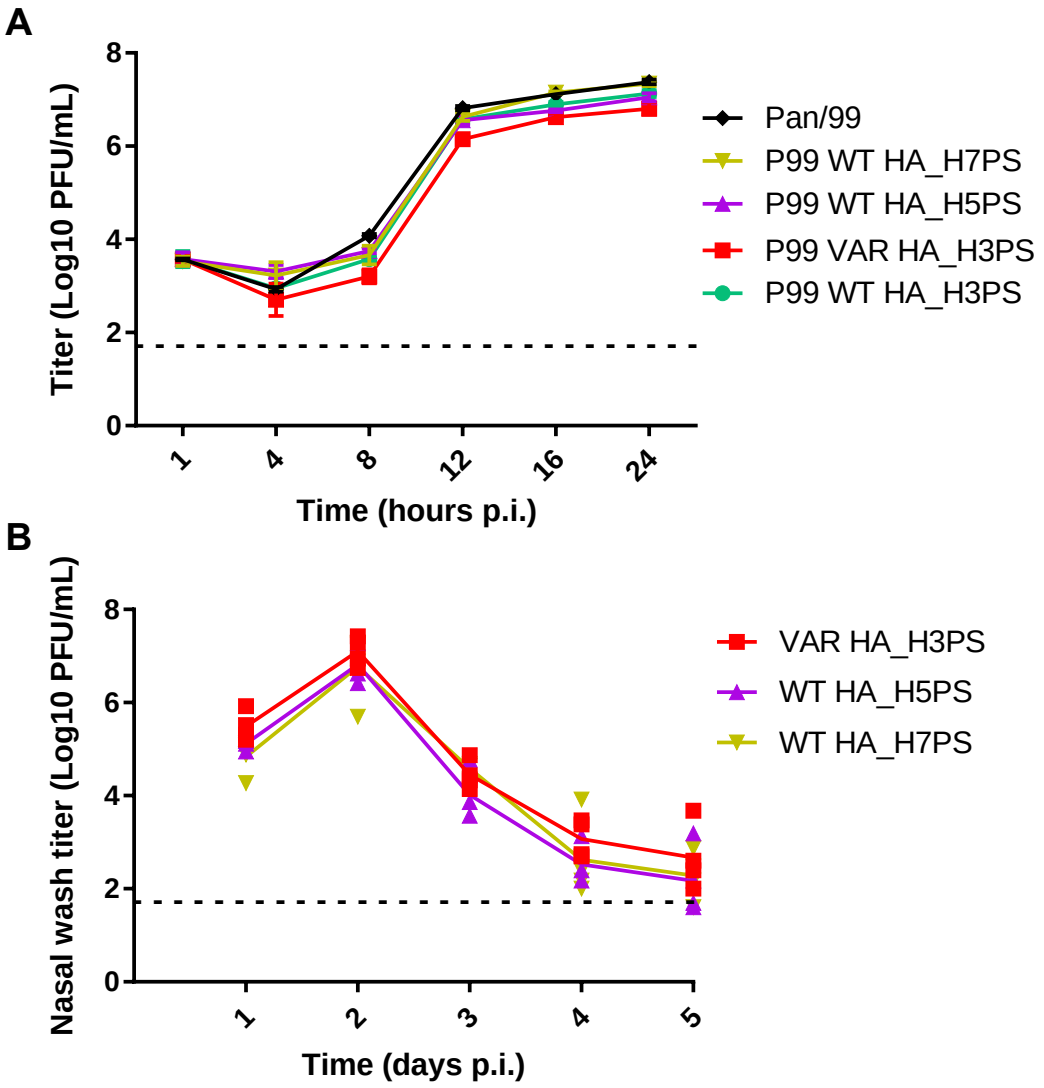

Fig. S3. The introduction of packaging signal regions to Pan/99 HA segments did not alter viral growth. (A) MDCK cells were infected at an MOI 5 PFU/cell and virus replication was allowed to proceed for a single cycle. Supernatants were sampled at 1, 4, 8, 12, 16, and 24 h p.i. and titered via plaque assay on MDCK cells. Colored lines represent Pan/99-based viruses with modified HA segments and the black line represents the control Pan/99 virus. N = 3 technical replicates per virus. Data are plotted as mean with SD. The limit of detection was 50 PFU/mL and is represented by a dotted black line. (B) Guinea pigs were inoculated intranasally with 5 x 10^5 PFU of virus and nasal lavage of each animal was performed daily on day 1 through day 5 p.i. Samples were titered via plaque assay on MDCK cells. N = 4 animals per virus. Individual data points are plotted and the means at each time point are connected. Analysis of the data using two-way ANOVA with Tukey's multiple comparisons revealed no significant differences across viruses. The limit of detection was 50 PFU/mL and is represented by a horizontal dotted black line. Values below the limit of detection were plotted as 25 PFU/mL.

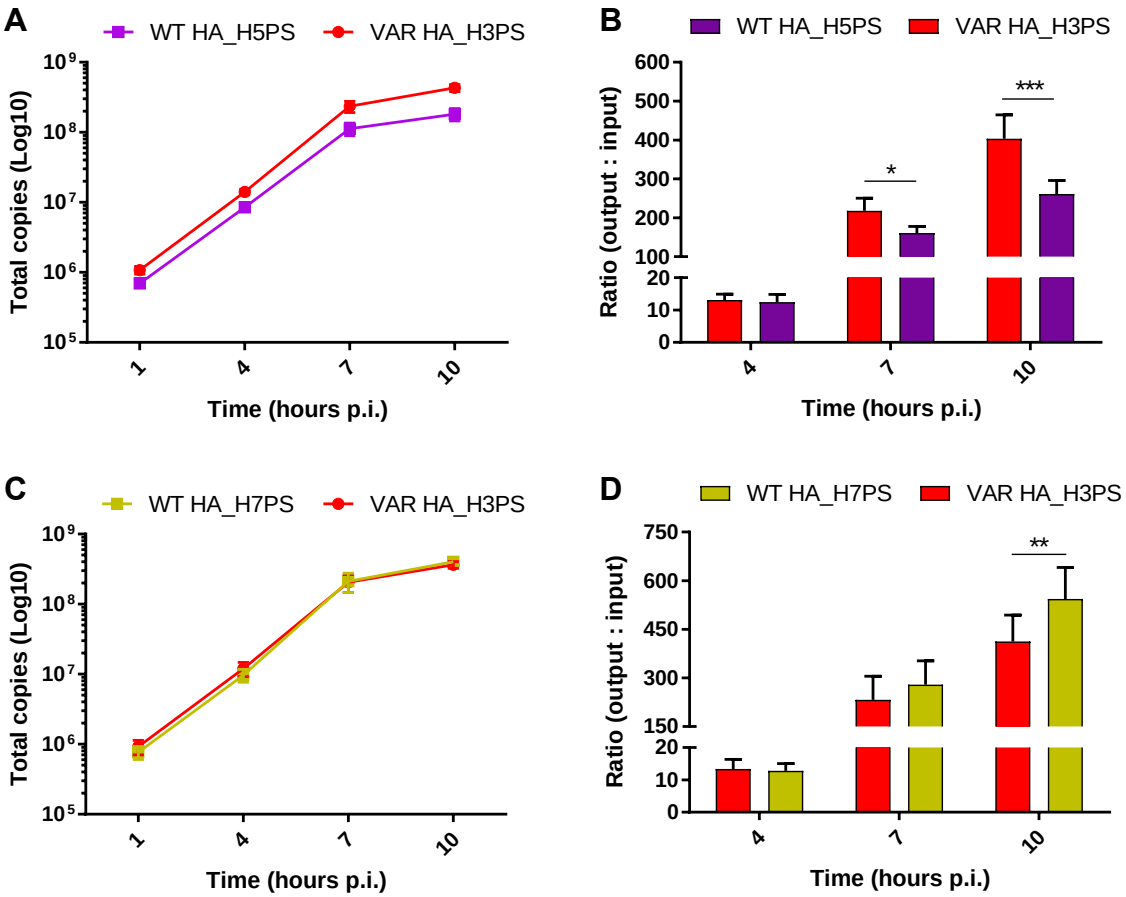

Fig. S4. Chimeric Pan/99 HA segments were replicated efficiently. MDCK cells were co-infected at a high MOI with HA_H3PS plus HA_H5PS viruses (A and B) or HA_H3PS plus HA_H7PS viruses (C and D). At 1, 4, 7, and 10 h p.i., cells and supernatants were harvested as a single sample and RT ddPCR was performed to enumerate total copies of each HA segment over time. Primers used were specific for the 3' HA packaging signal region of each segment. N = 6 (two biological replicates performed in triplicate with ddPCR performed in duplicate for all). (B and D) HA segment replication over time was normalized to input virus by dividing the 4, 7, and 10 h output values by the 1 h input values and resulting ratios were graphed; ***p < 0.0001, **p = 0.0058, *p = 0.0158 using two-way ANOVA with Sidak's multiple comparisons. All data are plotted as mean with SD.

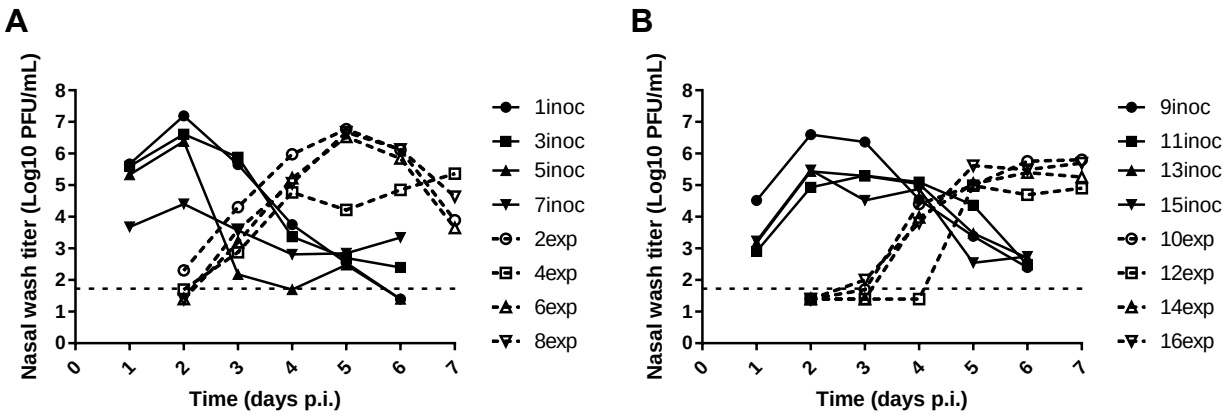

Fig. S5. Guinea pigs co-infected with modified HA viruses supported robust virus growth and transmission to naïve contacts. Guinea pigs were co-infected with either HA_H3PS plus HA_H5PS viruses (A) or HA_H3PS plus HA_H7PS viruses (B) and nasal lavage was performed daily up to day 6 p.i. and titered via plaque assay on MDCK cells (solid black curves). At day 1 p.i., a naïve contact animal was co-housed with each inoculated animal. Nasal lavage of exposed (exp) animals was performed daily day 2 - day 7 p.i. and titered via plaque assay on MDCK cells (dotted black curves). Each line represents one animal, N = 4 for each condition. The limit of detection was 50 PFU/mL and is represented by a horizontal dotted black line. Values below the limit of detection were plotted as 25 PFU/mL.

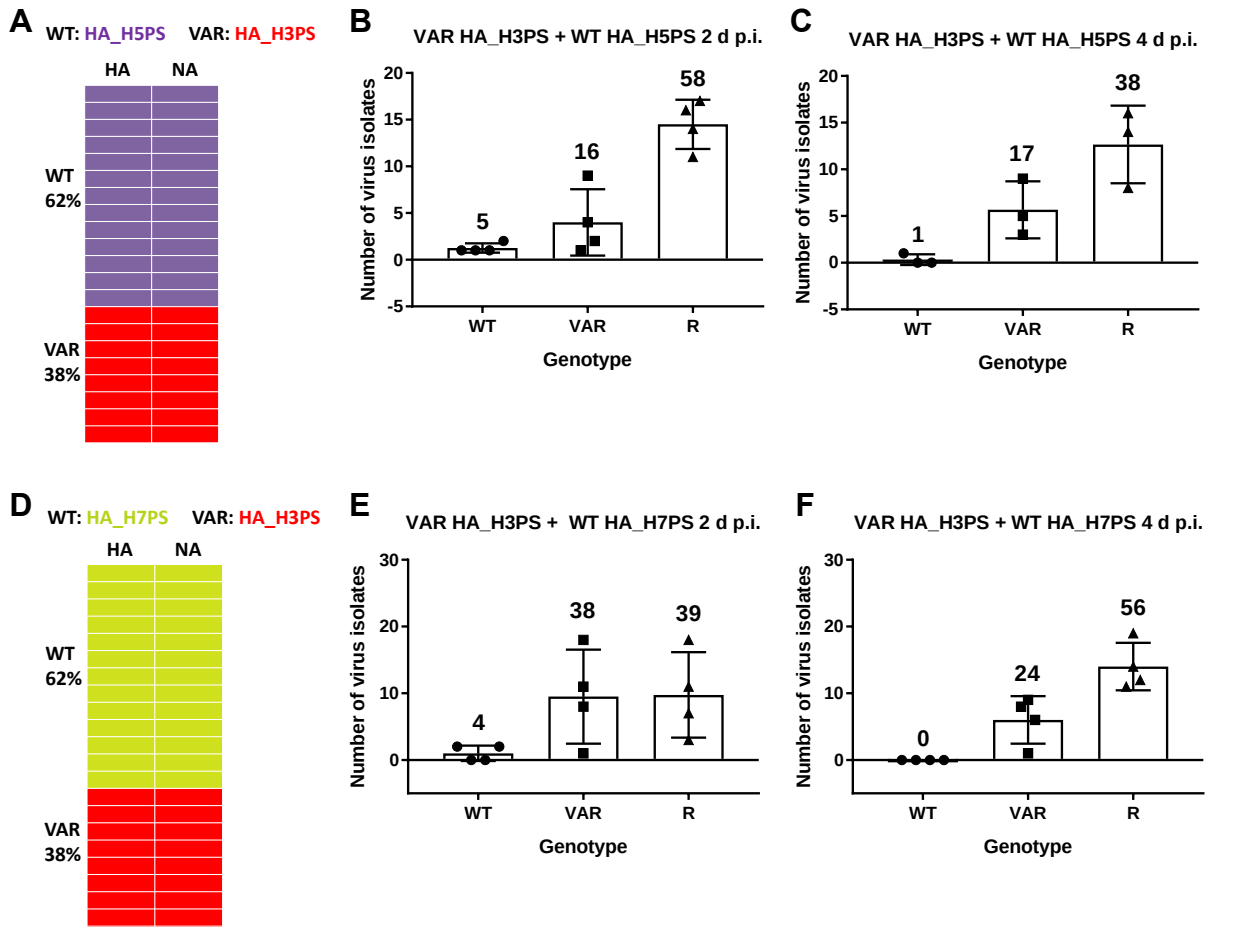

Fig. S6. The VAR virus strongly predominated over WT in nasal lavage samples despite slight overrepresentation of WT in the inocula. Guinea pigs were inoculated with a WT:VAR virus mixture consisting of HA_H3PS plus HA_H5PS (A-C) or HA_H3PS plus HA_H7PS viruses (D-F). The input virus was genotyped (A and D) as well as virus isolates from the inoculated animals taken at 2 d p.i. (B and E) and 4 d p.i. (C and F). Values are plotted as mean with SD, with total virus isolates identified as each genotype stated above the mean. Each data point represents one animal: N = 4 animals for (B); N = 3 animals for (C), N = 4 animals for (E); N = 4 animals for (F). N = 21 virus isolates for (A and D); N = 79 virus isolates for (B); N = 56 virus isolates for (C); N = 81 virus isolates for (E); N = 80 virus isolates for (F). The reassortant isolates in (B, C, E, and F) are the same isolates characterized in Fig. 3. All data originate from Fig. 2. WT = parental WT genotype, VAR = parental VAR genotype, R = any reassortant genotype.

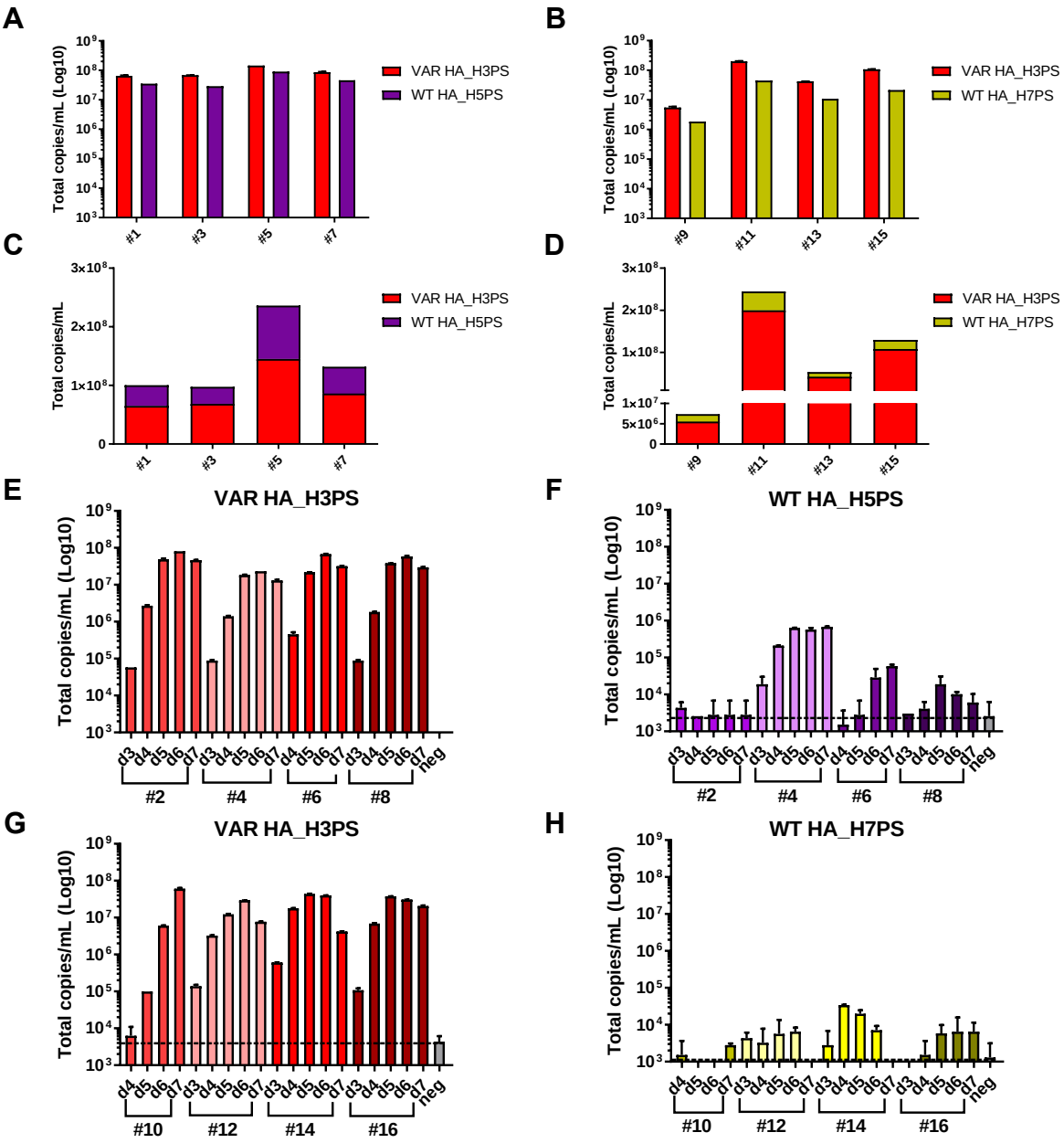

Fig. S7. HA segments carrying H5, but not H7, packaging signals were transferred robustly to a subset of naïve contact animals. Nasal wash fluid of co-infected (donor) animals on day 2 p.i (A-D) or contact animals exposed to co-infected guinea pigs (E-H) was analyzed by RT ddPCR to evaluate HA segment presence. Primers specific to the 3' packaging signal of each HA segment were used, and each reaction was performed in duplicate. (A-D) HA segment expression on day 2 p.i. of guinea pigs inoculated with HA_H3PS plus HA_H5PS viruses (A and C) or HA_H3PS plus HA_H7PS viruses (B and D). N = 4 animals per condition. Data are plotted as mean with SD (A and B) or as grouped means for each segment (C and D). Guinea pig ID numbers are indicated on the X axis. (E-H) HA segment transmission to animals exposed to HA_H3PS plus HA_H5PS co-infected animals (E and F) or HA_H3PS plus HA_H7PS co-infected animals (G and H). The day of sampling is shown on the X axis and guinea pig ID numbers are indicated underneath. The background values of the assay (measured using primer + dH2O) were used to define the negative (neg), which is represented by a dotted black line. For (E), the background values were zero and therefore the dotted line does not appear on the graph. Data are plotted as mean with SD.

Table S1. Primers used for HRM analysis

Segment	*Forward Primer*	*Reverse Primer*
Pan/99 PB2	TGGAATAGAAATGGACCTGTGA	GGTTCCATGTTTTAACCTTTCG
Pan/99 PB1	AGGCTAATAGATTTCCTCAAGGATG	ACTCTCCTTTTTCTTTGAAAGTGTG
Pan/99 PA	TGCAACACTACTGGAGCTGAG	CTCCTTGTCACTCCAATTTCG
Pan/99 HA	CCTTGATGGAGAAAACTGCAC	CAACAAAAAGGTCCCATTCC
Pan/99 NP	CAACATACCAGAGGACAAGAGC	ACCTTCTAGGGAGGGTCGAG
Pan/99 NA	TCATGCGATCCTGACAAGTG	TGTCATTTGAATGCCTGTTG
Pan/99 M	GTTTTGGCCAGCACTACAGC	CCATTTGCCTGGCCTGACTA
Pan/99 NS	ACCTGCTTCGCGATACATAAC	AGGGGTCCTTCCACTTTTTG

Table S2. Primers used for ddPCR

Segment	*Forward Primer*	*Reverse Primer*	*Figure*
PB2 Terminal	GGAACCTGATGTCGCAGTCT	CTTTTCCTGTCTCCCTGATG	*Fig. 5*
Pan/99 HA	CCTTGATGGAGAAAACTGCAC	CAACAAAAAGGTCCCATTCC	*Fig. 5*
Pan/99 PB2	TGGAATAGAAATGGACCTGTGA	GGTTCCATGTTTTAACCTTTCG	*Fig. 5*
HA Internal	TAAGGGGTGTCTCCAGCAGA	CCAATGGGTGCATCTGACCT	*Fig. 5*
PB1 Terminal	CCTAAAGGTTCCAGCGCAAA	TGTGTCCATGGTGTACCCTG	*Fig. 5*
PA Terminal	CAACCCGATGATTGTCGAAC	CAAGTGAGTGCATATTGCTGC	*Fig. 5*
NP Terminal	GCGTCCCAAGGCACCAAAC	CCATCAATCATCTTCCCGAC	*Fig. 5*
NA Terminal	GGCTCTGTTTCTCTCACTATTG	GGGAGTTGCATTCATATTGCTTG	*Fig. 5*
M Terminal	CGTATGTTCTCTCTATCGTTCC	GAGAGCCTCAAGATCTGTG	*Fig. 5*
NS Terminal	CTTTGGCATATCCGGAAGCAAG	GCTTTGATGTCTAGACCGAG	*Fig. 5*
HA_PSmut	CACTTTCCTATATCCTTTGCCTC	CCGTTTGACACTGCATGG	*Fig. S2*
HA VAR	CTTTGAGCTACATTTTATGTCTGG	CCGTTTGACACTGCATGG	*Fig. S2*
HA_H3PS	AAGACTATCATTGCTTTGAGCTAC	GTTGCCGTGCTGTTGTAAT	*Fig. S4*
HA_H5PS	TAGTGCTTCTTCTTGCAATAGT	TGCTCTGTCGAGTTGTTTG	*Fig. S4*
HA_H7PS	TCAAATCCTGGTATTCGCT	CGTTTGACACGGAATGATG	*Fig. S4*

References

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2. Fodor E, *et al.* (1999) Rescue of Influenza A Virus from Recombinant DNA. *J Virol* 73(11):9679-9682.