Egg-adaptation pathway of human influenza H3N2 virus is contingent on natural evolution

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ABSTRACT

Egg-adaptive mutations in influenza hemagglutinin (HA) often emerge during the production of egg-based seasonal influenza vaccines, which contribute to the largest share in the global influenza vaccine market. While some egg-adaptive mutations have minimal impact on the HA antigenicity (e.g. G186V), others can alter it (e.g. L194P). Here, we show that the preference of egg-adaptation pathway in human H3N2 HA is strain-dependent. In particular, Thr160 and Asn190, which are found in many recent H3N2 strains, restrict the emergence of L194P but not G186V. Our results further suggest that natural amino acid variants at other HA residues also play a role in determining the egg-adaptation pathway. Consistently, recent human H3N2 strains from different clades acquire different mutations during egg passaging. Overall, these results demonstrate that natural mutations in human H3N2 HA can influence the egg-adaption pathway, which has important implications in seed strain selection for egg-based influenza vaccine.

Keywords: Influenza, H3N2, egg-based vaccine, mutation, epistasis, evolution

INTRODUCTION

Vaccination is considered the most effective approach to prevent influenza infection. Seasonal influenza vaccine offers protection against two influenza A subtypes, namely H1N1 and H3N2, as well as influenza B viruses. However, the vaccine effectiveness against H3N2 is typically lower than H1N1 and influenza B [1-3]. Besides vaccine mismatch in certain influenza seasons, the occurrence of egg-adaptive mutations during vaccine production can also lead to the reduction of vaccine effectiveness [4-6]. When grown in eggs, human H3N2 viruses often acquire mutations in the receptor-binding site (RBS) of hemagglutinin (HA) to facilitate the binding to α 2,3-linked sialic acid, which is an avian-type receptor [7-9]. Since HA RBS partially overlaps with several major antigenic sites [10], some egg-adaptive mutations can alter the antigenicity of HA [4, 5, 9, 11-13].

G186V and L194P are the two most common egg-adaptive mutations in H3N2 HA [14]. Although both mutations are in the RBS, L194P but not G186V significantly alters the antigenicity of HA [15]. Thus, identifying factors that influence the preference of egg-adaptation pathway can help optimize the effectiveness of egg-based influenza vaccine. Our previous study has demonstrated a strong incompatibility between G186V and L194P, in which their co-occurrence is extremely deleterious to the virus [14]. This epistatic interaction suggests that G186V and L194P represent two mutually exclusive egg-adaptation pathways. Given that epistasis is pervasive in the HA RBS and that HA RBS is constantly evolving [13, 16-19], it is possible that different strains have different preferences of egg-adaptation pathways. However, it remains to be explored whether such differential preference exists.

In this study, sequence analysis and mutagenesis experiments are used to identify amino acid variants on human H3N2 HA that can affect the preference of egg-adaptation pathway. We discover that L194P is incompatible with T160 and N190, both of which are prevalent in recent human H3N2 strains. Consistently, recent human H3N2 strains are incompatible with L194P. Our results further indicate that amino acid variants at other residues also contribute to the

L194P incompatibility in recent human H3N2 strains. Along with the observation that human H3N2 strains from different clades prefer different egg-adaptation pathways, we conclude that the preference of egg-adaptation pathway changes as human H3N2 virus evolves.

RESULTS

Differential egg-adaptation pathways in historical vaccine strains

Our previous study has shown that the two major egg-adaptive mutations G186V and L194P are incompatible [14]. To examine whether different human H3N2 strains have different preferences of egg-adaptation pathways, HA sequences from egg-passaged strains were collected from the Global Initiative for Sharing Avian Influenza Data (GISAID) [20] (**Supplementary Table 1**). Specifically, we focused on egg-passaged strains that were derived from WHO-recommended candidate vaccine viruses for influenza seasons between 2008 and 2020. Egg-passaged strains that were derived from different parental viruses have acquired different egg-adaptive mutations (**Figure 1A**). For example, all egg-passaged strains from A/Victoria/361/2011, A/Texas/50/2021, A/Switzerland/9715293/2013, and A/Hong Kong/2671/2019 have acquired G186V, whereas those from A/Hong Kong/4801/2014, A/Singapore/INFIMH-16-0019/2016 (Sing16) and A/Switzerland/8060/2017 (Switz17) have acquired L194P. This observation is consistent with the hypothesis that the preference of egg-adaptation pathway is strain dependent.

To further test our hypothesis, we measured the replication fitness of G186V and L194P in Sing16, Switz17, and A/Kansas/14/2017 (Kansas17) (**Supplementary Table 2**). Our mutagenesis experiments were based on egg-adapted strains. As a result, all the Kansas17 mutants in this experiment were constructed in the presence of the background mutations D190N and S219Y, which were present in 100% (15/15) and 87% (13/15), respectively, of egg-passaged Kansas17 strains in GISAID. Similarly, all the Sing16 and Switz17 mutants were constructed in the presence of the background mutation T160K, which was present in all egg-passaged strains derived from Sing16 and Switz17 in GISAID. The fitness effects of

four different combinations of amino acid variants at residues 186 and 194 were tested, namely 1) G186/L194, which represented the amino acid variants in the unpassaged strains, 2) V186/L194, which represented the single egg-adaptive mutation G186V, 3) G186/P194, which represented the single egg-adaptive mutation L194P, and 4) V186/P194, which represented the co-occurrence of two incompatible egg-adaptive mutations [14].

As expected, all variants with G186/L194 could be rescued, whereas all variants with V186/P194 had no detectable titer (**Figure 1B**). In contrast, the fitness effects of V186/L194 and G186/P194 were different in different strains. Although V186/L194 could be rescued in all three strains (**Figure 1B**), it only facilitated the egg-adaptation of Kansas17-D190N/S219Y, but not Sing16-T160K and Switz17-T160K (**Figure 1C**). Similarly, G186/P194 could only be rescued in Sing16-T160K and Switz17-T160K, but not Kansas17-D190N/S219Y. Next-generation sequencing further showed that during egg-passaging, V186/L194 were stable in Kansas17-D190N/S219Y, and G186/P194 were stable in both Sing16-T160K and Switz17-T160K (**Figure 1D and Supplementary Table 3**). These results substantiate that there are differential preferences of egg-adaptation pathways among different strains.

Residues 160 and 190 influence the egg-adaptation pathway

The inability of rescuing G186/P194 in Kansas17-D190N/S219Y suggests that G186V is not the only mutation that is incompatible with L194P. We therefore aimed to identify additional mutations that were incompatible with L194P. The HA sequence of Kansas17 X-327, which was an egg-adapted strain with G186V, was compared to that of Switz17 NIB-112 and Sing16 NIB-104, which were two egg-adapted strains with T160K/L194P. Of note, the HA sequences are equivalent between Kansas17 X-327 with V186G/L194P (**Figure 2A**) and the unrescuable Kansas17-G186/P194/N190/Y219 (see above, **Figure 1B**). This sequence analysis led us to identify four candidate amino acid variants in the RBS that might be incompatible with L194P, namely S159, N190, S193 and Y219. Subsequently, S159Y, N190D, S193F and Y219S, which represented the RBS mutations from Kansas17 X-327 to Switz17 NIB-112 and Sing16 NIB-

104, were introduced individually into the HA of Kansas17 X-327 in the presence of the background mutations V186G and L194P. V186G was included in the background since V186 and P194 were known to be incompatible [14]. Our virus rescue experiment showed that the fitness defect of V186G/L194P in Kansas17 X-327 could be restored by N190D (**Figure 2B**). Additionally, Kansas17 X-327 with and without V186G/L194P/N190D had comparable replication fitness in eggs (**Figure 2C**). Next-generation sequencing further confirmed that Kansas17 X-327 with V186G/L194P/N190D was genetically stable during egg-passaging (**Figure 2D and Supplementary Table 3**). To examine if the incompatibility between N190 and P194 could be generalized to other strains, we further introduced D190N into Switz17-K160/P194 and Sing16-K160/P194, which were equivalent to Switz17 NIB-112 and Sing16-K160/P194, respectively. Consistent with the results for Kansas17 X-327, D190N was deleterious in Switz17-K160/P194 and Sing16-K160/P194 (**Figure 2E**). Together, these results demonstrate the incompatibility between N190 and P194.

During our sequence analysis, we noticed that the egg-adaptive mutation T160K [5] often co-occurred with L194P, as exemplified by Switz17 NIB-112 and Sing16 NIB-104. Our rescue experiment showed that T160 was incompatible with P194 in both Sing16 and Switz17 (**Figure 2E**). As a result, in addition to V186 and N190, T160 is another amino acid variant that is incompatible with P194.

Recent human H3N2 strains are incompatible with L194P

Among the amino acid variants that are incompatible with P194, T160 and N190 have reached high occurrence frequency in recent human H3N2 HA. T160, which introduced an N-glycosylation site at N158, was fixed in clade 3C.2a during the 2014-2015 influenza season [5], whereas N190 emerged more recently along with I160 and D186 and reached >90% in 2021 (**Figure 3A**). Not surprisingly, A/Victoria/22/2020 (Vic20) and A/Italy/11871/2020 (Italy20), which were both from clade 3C.2A1b.1a and had T160/N190, could not be rescued in the presence of L194P (**Figure 3B**). In contrast, introducing V186 into Italy20 yielded high titer in

both virus rescue and egg-passaging experiments (**Figure 3C**). These observations illustrate that recent human H3N2 strains have a strong preference against L194P as the egg-adaptation pathway.

While we have shown above that T160 and N190 were incompatible with L194P in Kansas17, Switz17, and Sing16 (**Figure 2**), Italy20 with T160K/N190D was still incompatible with L194P (**Figure 3D**). This result indicates that besides T160 and N190, additional amino acid variants in Italy20 were also incompatible with L194P. We postulated that those additional amino acid variants could be identified by comparing the HA sequences of Italy20 and Sing16, since Sing16 was compatible with L194P in the presence of K160/D190 (**Figure 2E**). Eight mutations in the receptor binding domain (residues 117-265) [21] were identified between Sing16 and Italy20 (**Figure 3E**). Based on spatial proximity between these eight mutations and L194P, we hypothesized that the differential fitness effects of L194P in Sing16 and Italy20 could be due to the presence of mutations G186D, F193S, and S198P in Italy20. Since the compatibility with L194P in Kansas17 was not influenced by the presence of S193 as shown in our rescue experiment above (**Figure 2A-B**), we focused on D186 and P198 here. However, even with mutations D186G and P198S, Italy20-T160K/D186G/N190D/P198S/L194P still could not be rescued (**Figure 3D**). Structural modeling further showed that none of the eight mutations from Sing16 to Italy20 increased the height of the RBS (**Figure 3F**), which was the mechanistic basis of incompatibility between L194P and G186V [14]. Together, these results demonstrate that the L194P incompatibility in recent human H3N2 strains is conferred by multiple amino acid variants including T160 and N190, and likely involves a mechanism that is different from G186V.

Egg-adaptation pathway is clade dependent

The recent emergence of L194P incompatibility suggests that the preference of egg-adaptive pathway has been evolving. To understand the preference of egg-adaptation pathway in recent human H3N2 strains, we analyzed the HA sequences of 72 egg-passaged H3N2 strains

from years 2019 to 2021, which belong to antigenically distinct clades [22-24]. By comparing the HA sequences between the egg-passaged strains and their non-egg-passaged counterparts, we observed that egg-adaptive mutations often emerged at residues 160, 186, 190, 194, 219 and 225 (**Figure 4 and Supplementary Table 4**). In addition, different clades showed different preferences of egg-adaptation pathways. This differential preference could be exemplified by residues 186 and 225. While all non-egg-passaged strains from both clades 3C.2a1b.1a and 3C.2a1b.2a2 had D186, egg-adaptive mutation D186N was common in 3C.2a1b.2a2 but not observed in 3C.2a1b.1a. Similarly, although D225 was present in all non-egg-passaged strains across all clades, egg-adaptive mutation D225G was prevalent in 3C.2a1b.2a2 but not observed in clades 3C.2a1b.2a, 3C.2a1b.2b, and 3C.2a1b.1b. These observations are consistent with the notion that the preference of egg-adaptation pathway in human H3N2 HA is influenced by natural mutations.

DISCUSSION

Egg-adaptation of influenza virus has been central to the production of seasonal influenza vaccine. Our work here shows that the preference of egg-adaptation pathway is strain dependent, due to epistasis between egg-adaptive mutations and natural amino acid variants. Specifically, we identified two natural amino acid variants T160 and N190 that are incompatible with the egg-adaptive mutation L194P. Our results also indicate that additional amino acid variants are responsible for the L194P incompatibility in recent human H3N2 HA. Since L194P is just one of many egg-adaptive mutations in human H3N2 HA, we are likely still far from comprehending the sequence determinants that influence the preference of egg-adaptation pathway.

Our previous studies have shown that epistasis is pervasive between mutations in the HA RBS [16-18]. One interesting observation is that epistasis between two HA RBS mutations can be modulated by a third mutation. For example, epistasis between the engineered mutations L226R and S228E in an H1 HA could only be observed in the presence of G225E [16]. This

type of higher-order epistasis may play a role in determining the egg-adaptation pathway. Despite the incompatibility between T160 and P194 in Sing16 and Switz17 (**Figure 2E**), we noticed the co-occurrence of T160 and P194 in four egg-adapted H3N2 strains (EPI1588456, EPI1584616, EPI1526498 and EPI1440496), which are all belong to clade 3C.2a1b.2b (**Figure 4 and Supplementary Table 4**). This observation implies that the incompatibility between T160 and P194 can be masked by other amino acid variants. As shown by a recent study, epistasis can also alter the antigenic effect of a given mutation in the HA RBS [25]. The complexity of epistasis that involves egg-adaptive mutations as well as the underlying biophysical mechanisms and the antigenic consequences will need to be explored in future studies.

We anticipate that the preference of egg-adaptation pathway will keep changing as human H3N2 HA continues to evolve. As a result, future studies of egg-adaptive mutations in human H3N2 strains are warranted. Given that different egg-adaptive mutations can have different antigenic effects [5, 8, 9, 12, 15, 26-31], characterization of individual egg-adaptive mutations is critical for the selection of vaccine seed strains with minimal antigenic change. Nevertheless, egg-based influenza vaccine production is an 80-year-old technology that needs to be replaced by more advanced alternatives. An important step forward was the commercialization of cell-based and recombinant influenza vaccines, which have shown better effectiveness than egg-based influenza vaccines [32, 33]. In addition, a Phase 1/2 clinical trial of an mRNA seasonal influenza vaccine candidate has initiated recently [34]. Despite the potentially slow transition, these alternatives will provide an ultimate solution to the problems of egg adaptation.

MATERIALS AND METHODS

Sequence analysis

Full-length HA sequences of WHO-recommended H3N2 candidate vaccine virus strains from 2008 to 2020 were downloaded from the Global Initiative for Sharing Avian Influenza Data (GISAI; <http://gisaid.org>) [20]. Information for these sequences is shown in **Supplementary**

Table 1. Information for the full-length HA sequences of viruses from 2019 to 2021, with and without egg-passaging, is shown in **Supplementary Table 4**. Sequences were aligned by MAFFT version 7 (<https://mafft.cbrc.jp/alignment/software/>) with default setting [35]. Passaging history was determined by parsing the regular expression in FASTA headers as described [36]. The frequencies of amino acids were plotted using WEBLOGO (<https://weblogo.berkeley.edu/logo.cgi>) [37]. Phylogenetic tree was built using Maximum Likelihood (ML) method by Geneious Prime (Geneious), with 100 bootstraps.

Cell culture

Humanized Madin-Darby canine kidney (hMDCK) cells with higher and stable expression of human 2,6-sialtransferase were kindly provided by Professor Yoshihiro Kawaoka, the University of Tokyo [38]. Both hMDCK cells and human embryonic kidney (HEK) 293T cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 25 mM HEPES, and 100 U mL⁻¹ penicillin-streptomycin (PS).

Generation of recombinant mutants

All H3N2 viruses generated in this study were based on the influenza eight-plasmid reverse genetics system [39, 40]. The HA and neuraminidase (NA) genes of the strains of interest (**Supplementary Table 2**) were synthesized by Sangon Biotech, and cloned into the pHW2000 vector as previously described [14]. For HA, the ectodomain was from the strains of interest, whereas the noncoding region, N-terminal secretion signal, C-terminal transmembrane domain, and cytoplasmic tail were from H1N1 A/PR/8/34 (PR8). For NA, the entire coding region was from the strains of interest, whereas the noncoding region of NA was from PR8. Mutations were introduced into the constructs by polymerase chain reaction (PCR). Primers used in this study were produced by Integrated DNA Technologies (Integrated DNA Technologies). Recombinant chimeric 6:2 reassortant viruses with the six internal genes (PB2, PB1, PA, NP, M, and NS) from PR8 were rescued by transfecting a co-culture of HEK 293T and hMDCK cells at 6:1 ratio with 70% confluence in a 6-well plate. Transfection was

performed using 16 μ L of TransIT-LT1 (Mirus Bio) and 1 μ g each of the eight plasmids encoding the corresponding influenza virus segments (a total amount of 8 μ g of DNA). At 6 h post-transfection, medium was replaced with 1 mL of MEM. At 24 h post-transfection, another 1 mL of MEM supplemented with 1 μ g mL⁻¹ tosylphenylalanyl chloromethyl ketone (TPCK)-trypsin was added. Viruses were harvested at 72 h post-infection and inoculated into hMDCK cells at 90% confluence in a T75 flask. Viruses were harvested when the cytopathic effect (CPE) was observed. Virus titer was determined by titration in hMDCK cells as previously described [41, 42].

Virus passaging in embryonic eggs

Embryonic eggs at 10 days post-fertilization were used for the virus passaging. For each passage, 10⁴ TCID₅₀ of virus was inoculated into one egg. The only exception was for the next-generation sequencing experiment (see below), 10⁶ TCID₅₀ was used for the first passage of Sing16-K160/V186/L194. The allantoic fluid was harvested at 48 h post-infection. Virus titer was determined by titration in hMDCK cells as previously described [41, 42]. Each experiment consisted of five consecutive passaging and was performed in triplicate.

Next-generation sequencing of egg-passaged viruses

The viral RNA of the egg-passaged virus was extracted using QIAamp Viral RNA Mini Kit (Qiagen). The extracted RNA was then reverse transcribed to cDNA using ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs). Subsequently, PCR was performed using PrimeSTAR Max DNA Polymerase (Takara) with the cDNA as template and virus-specific primers 5'-CAC TCT TTC CCT ACA CGA CGC TCT TCC GAT CT NNN GGT CAC TAG TTG CCT CAT CCG G-3' and 5'- GAC TGG AGT TCA GAC GTG TGC TCT TCC GAT CTG GTG CAT CTG ATC TCA TTA TTG-3'. A second round of PCR was performed to add the rest of adapter sequence and index to the amplicon using primers 5'- AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT-3' and 5'- CAA GCA GAA GAC GGC ATA CGA GAT XXX XXX GTG ACT GGA GTT CAG ACG TGT GCT-3'. Nucleotides in primers

annotated by an 'N' or "X" represented the index sequence for distinguishing PCR products derived from different viruses, passages, and biological replicates. The final PCR products were sequenced by Illumina MiSeq PE300.

Analysis of next-generation sequencing data

Sequencing data was obtained in FASTQ format and analyzed using in-house Python and shell scripts. Briefly, paired-end reads were merged by PEAR [43]. The merged reads were then parsed by SeqIO module in BioPython and translated into protein sequences [44]. For each sample, the frequency of amino acid variant i was computed as follows:

$$frequency_i = \frac{read\ count_i}{sequencing\ depth}$$

where $read\ count_i$ represents the number of reads that contain amino acid variant i and $sequencing\ depth$ represents the total number of reads in the sample of interest.

Structural modeling

Residues 57 to 261 of a monomer of A/Michigan/15/2014 (H3N2) influenza virus HA (PDB 6BKT) [17] was extracted. The PDB file was renumbered using pdb-tools [45]. Point mutagenesis for T128A, V130I, T135K, A138S, G186D, G186V, D190N, F193S, L194P and S198P (all in original numbering) were performed using the fixed backbone (fixbb) backbone design application in Rosetta (RosettaCommons). For each replicate, one hundred poses were generated and the lowest scoring pose was used for downstream analysis. The fixbb application for each mutation was performed in triplicate. A constraint file for each lowest scoring pose was obtained using the minimize_with_cst application in Rosetta and converting the log file to a constraint (cst) file using the convert_to_cst_file application in Rosetta. Subsequently, fast relax was performed on wild type or mutant using the relax application in Rosetta with the corresponding constraint file [46]. For each replicate, 30 poses were generated and the lowest scoring pose was used to measure the height of the RBS, which is the distance between the side chain oxygen atom of Y98 (H3 numbering) and Cα of amino acid 190 (H3 numbering) using PyMOL (Schrödinger).

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325 **Data availability**

326 Raw sequencing data have been submitted to the NIH Short Read Archive under accession
327 number: BioProject PRJNA800806. Codes for analyzing next-generation sequencing data
328 have been deposited to https://github.com/Wangyiquan95/HA_egg_passage. Code and
329 source files for structural modeling using Rosetta have been deposited to
330 https://github.com/timothyjtan/HA-RBD_height.

331

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340

341 **AUTHOR CONTRIBUTIONS**

342 W.L., H.L., C.K.P.M., and N.C.W. conceived and designed the study. W.L. and Y.S. performed
343 the experiments. W.L., T.J.C.T., and Y.W. analyzed the data. W.L., R.B., C.K.P.M., and N.C.W.
344 wrote the paper, and all authors reviewed and edited the paper.

345

346 **DECLARATION OF INTERESTS**

347 The authors declare no competing interests.

Figure 1. Differential preference of egg-adaptive mutations in human H3N2 vaccine strains. (A) Frequencies of mutations among different egg-passaged seasonal H3N2 vaccine strains are shown as sequence logos. Amino acid variants from residues 183 to 196 are shown (H3 numbering). The number of egg-passaged strains included in this analysis is indicated in the parenthesis. The relative size of each amino acid letter represents its frequency in the sequences. Grey letters represent the amino acid variants that are observed in the corresponding unpassaged parental strains. (B) Replication fitness of different mutants of human H3N2 vaccine strains was examined in a virus rescue experiment. Viral titers were

measured by TCID₅₀. **(C)** The viral titer of each human H3N2 vaccine strain with either V186/L194 or G186/P194 was measured during egg-passaging. **(B-C)** Results are shown as means $\pm$ SD of three independent experiments. The dashed line represents the lower detection limit. Amino acid variant representing an egg-adaptive mutation is underlined. **(D)** Frequencies of mutations in the receptor-binding subdomain (residues 117-265) [21] that emerged during serial passaging in eggs. The strain of inoculum for each passaging experiment is indicated above each plot, with those representing egg-adaptive mutations underlined. Frequencies are shown as means of three biological replicates. Only those mutations that reached a minimum average frequency of 10% after the fifth passage are plotted.

Figure 2. Epistatic interactions that involve egg-adaptive mutations. (A) Amino acid variants from residues 150 to 220 among the indicated strains are shown. The rest of the sequences from residues 150 to 220 were completely conserved among these strains. Major egg-adaptive mutations G186V and L194P are in yellow. Amino acid variants that are unique to Kansas17 strains are in red while variants unique to Switz17 and Sing16 are in blue. Of note, Kansas17 X-327, Switz17 NIB-112, and Sing16 NIB-104 are egg-adapted strains, whereas Kansas17 X-327 V186G/L194P (red) was a mutant generated in this study. (B) Replication fitness of Kansas17 X-327 with different mutations was examined in a virus rescue experiment. (C) The replication fitness of Kansas17 X-327 and Kansas17 X-327 with triple mutations (V186G/L194P/N190D) in eggs were examined during serial egg-passaging. (D) Frequencies of mutations in the receptor-binding subdomain (residues 117-265) [21] that emerged during egg-passaging of the indicated strains. Results are shown as means of three independent biological replicates. Only those mutations that reached a minimum average frequency of 10% after the fifth passage are plotted. (E) Replication fitness of Sing16 and Switz17 L194P virus with or without K160T and D190N mutations was assessed in a virus rescue experiment. (B,

384 **C, E)** All viral titers were measured by TCID₅₀. Results are shown as means ± SD of three
385 independent experiments. The dashed line represents the lower detection limit.

Figure 3. Incompatibility between N190 and P194. (A) Frequencies of amino acid variants at residues 160, 186 and 190 in human H3N2 HA over time are shown. Only those variants that reached a maximum annual frequency of 35% are plotted. (B) Two strains in clade 3C.2a1b.1a (Vic20 and Italy20) with or without L194P mutation was examined in a virus rescue experiment in hMDCK cells. Of note, most unpassaged strains in clade 3C.2a1b.1a, including both Vic20 and Italy20, naturally contain N190. (C) The replication fitness of Italy20 with different mutations was examined in a virus rescue experiment (hMDCK) as well as in an egg-passaging experiment, in which 10⁴ TCID₅₀ of the rescued viruses were propagated in eggs

for 48 hours. **(D)** The replication fitness of Italy20 with different mutants was measured in a virus rescue experiment. **(B-D)** Viral titers were measured by TCID₅₀. Results are shown as means $\pm$ SD of three independent experiments. The dashed line represents the lower detection limit. **(E)** The locations of mutations between Sing16 and Italy20 in the receptor-binding subdomain (residues 117-265) [21] are shown in blue (PDB 6BKT) [17]. L194P is in orange. Sialylated glycan receptor is in yellow sticks representation. **(F)** The structural impact of each mutation was modeled by Rosetta [46]. The distance between the phenolic oxygen of Tyr98 (OH₉₈) and the C α of residue 190 was computed as the height of receptor-binding site (RBS). Three replicates, each with 100 simulations, were performed. Each data point represents the lowest scoring pose in a replicate. Error bars represent means $\pm$ SD. *P*-value was computed by two-tailed t-test. Only the difference between WT and G186V is statistically significant (*P* < 0.05).

Figure 4. Egg-adaptive mutations in recent human H3N2 HA. A phylogenetic tree was built using the HA sequences of 61 human H3N2 strains without egg-passaging (unpassaged strains). Different clades are highlighted in different colors. WHO-recommended vaccine strains are indicated by a red triangle symbol. These unpassaged human H3N2 strains correspond to the parental strains of 72 egg-passaged strains. Since multiple egg-passaged strains with different HA sequences could be generated from a single unpassaged strain, the number of unpassaged strains is less than that of egg-passaged strains. Frequencies of amino acids at HA residues 160, 186, 190, 194, 219 and 225 among egg-passaged strains in different clades are shown as sequence logos. The relative size of each amino acid letter represents its frequency in the sequences. For each clade, amino acid variants that are also observed in the unpassaged strains are in grey and listed below each sequence logo.

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