

# Deep mutational scanning of an H3 hemagglutinin can inform evolutionary forecasting of human H3N2 influenza virus

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## Abstract

Abstract text.

## INTRODUCTION

Very rough outline:

- Mutations are rampant in the evolution of human influenza virus. Seasonal H3N2 influenza virus in particular rapidly accumulates mutations.
- The evolution of H3N2 is also characterized by clade competition and population turnover.
- There have been efforts to predict evolutionary success.
- Mutations that contribute to antigenic evolution largely determine strain success.
- RNA viruses can accumulate deleterious mutations, and because the influenza does not appreciably recombine, deleterious mutations are linked to beneficial ones.
- Mutations in HA that impact viral growth may influence strain success.
- We need to understand the functional impact of mutations in HA.

- Previously, we measured the effect of all possible single amino-acid mutations to an H1 hemagglutinin from the A/WSN/1933 (H1N1) strain ([Thyagarajan and Bloom, 2014](#); [Doud and Bloom, 2016](#)).
- However, this is a highly lab-adapted strain, and the measurements in this strain may not be particularly relevant for studying mutational processes of more contemporaneous strains circulating in the human population.
- We chose to study the Perth/2009 H3 HA.
- This also enabled a comparison of how the preferences have shifted for two diverged HA's.

## RESULTS

### Deep mutational scanning of HA from a recent strain of human H3N2 influenza virus

We performed a deep mutational scan to measure the effects of all amino-acid mutations to the A/Perth/16/2009 (H3N2) HA on viral replication in cell culture. This strain was the H3N2 component of the influenza vaccine from 2010-2012. We used a variant of this HA that supported better viral replication in cell culture than did the sequence in GenBank [[Figure S1](#)]. This HA variant contained two mutations: G78D and T212I. The G78D mutation occurs at low frequency in natural H3N2 sequences, and T212 is a site where a mutation to Ala rose to fixation in human influenza in ~2011.

We mutagenized the entire gene at the codon level to create mutant plasmid libraries harboring an average of ~1.4 codon mutations per clone. We then generated mutant virus libraries from the mutant plasmids using a helper virus system that enables the efficient generation of complex virus libraries ([Doud and Bloom, 2016](#)) (Figure 1A). In order to reduce biosafety concerns and to maximize viral titers, we used an HA-deficient helper virus carrying WSN/1933 internal and NA genes to rescue the mutant viruses. Additionally, we used MDCK-SIAT1 cells constitutively expressing the TMPRSS2 protease, which facilitates HA cleavage and activation in the human airway ([Böttcher et al., 2006](#); [Böttcher-Friebertshäuser, et al., 2010](#)). All of the experiments were completed in full biological triplicate (Figure 1B). We also passaged and deep sequenced library 3 in technical replicate (denoted as library 3-1 and 3-2) to gauge to the amount of experimental noise occurring *within* a single biological replicate. As a control, we used the unmutated gene to generate and passage viruses carrying wildtype HA.

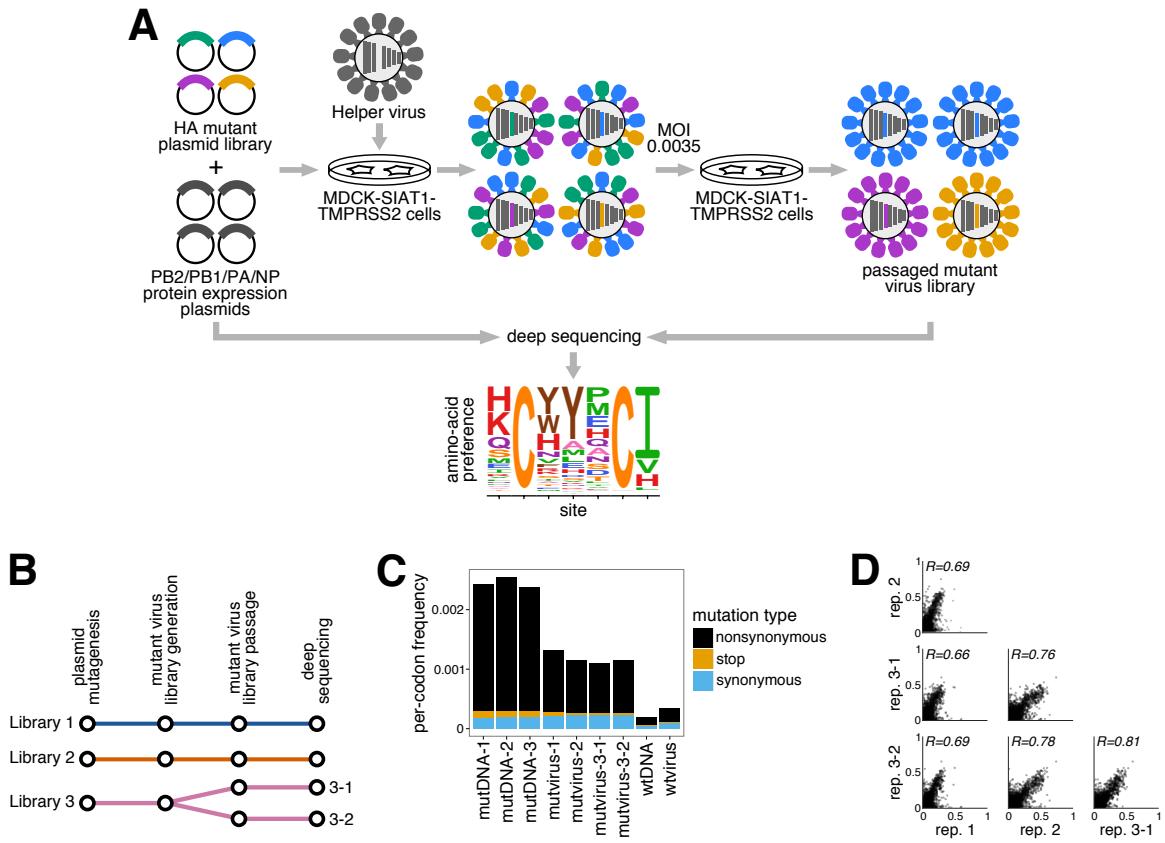
Deep sequencing revealed selection against non-functional HA variants as evidenced by the reduced mutation frequencies of the mutant viruses compared to their starting frequencies in the mutant plasmids (Figure 1C). Specifically, stop codons were purged to 20-45% of their original starting frequencies, after correcting for error rates from the wildtype controls. Although the majority of stop codons were purged, incomplete purging of stop codons is likely due to complementation of virions. We also observed purging of nonsynonymous mutations to 30-40% of their initial starting frequencies after error correction, suggesting strong selection against deleterious HA variants.

We next quantified the reproducibility of our deep mutational scanning measurements across biological and technical replicates. We inferred the amino-acid preferences at each site in the Perth/2009 HA using the method described in Bloom (2015) and implemented in the dms\_tools2 software [[https://jbloomlab.github.io/dms\\_tools2/](https://jbloomlab.github.io/dms_tools2/)]. These preferences represent an estimate of the 567 sites  $\times$  20 amino acids = 11340 experimental measurements and are normalized to sum to one at each site. The correlations of the amino-acid preferences between each pair of replicates is shown in Figure 1D. The biological replicates are fairly well-correlated, with a Pearson's  $R$  ranging from 0.69 to 0.78. Replicate 1 exhibits the least amount of correlation with the other biological replicates, consistent with the observation that this replicate showed the weakest selection against stop and nonsynonymous mutations and might therefore be subject to more experimental noise. Of note, the two technical replicates 3-1 and 3-2 were only slightly more reproducible than that between biological replicates. This suggests that bottlenecking of the virus library during the low MOI passage contributes to much of the noise observed in our experiments, as we are only able to passage a finite number of viral particles.

### The HA preferences improve phylogenetic fit of natural H3 sequences

How well do the Perth/2009 HA preferences inferred from experimental measurements describe the evolution of H3N2 influenza virus in nature? This question can be addressed by evaluating how well experimentally informed codon substitution models (ExpCM's) constructed from our laboratory measurements improve phylogenetic fit of H3N2 evolution (Hilton et al., 2017). The results in Table 1 show that the ExpCM outperforms conventional substitution models in describing the evolution of human H3N2 HA. The ExpCM also optimizes a stringency parameter ( $\beta$ ) for the preferences to more closely reflect the strength of selection in nature. The stringency parameter in the ExpCM is equal to 2.44, which indicates that although the same amino acids are preferred, the strength of selection is more stringent in nature than in our experiments. Figure 2 shows a logo plot of the Perth/2009 HA amino-acid preferences rescaled by this stringency parameter.

A closer examination of the logo plot reveals that the preferences generally agree with



**Figure 1: Deep mutational scanning experiments of H3 HA.** (A) We generated Perth/2009 HA mutant virus libraries in MDCK-SIAT1-TMPRSS2 cells using a helper virus approach described in [Doud and Bloom \(2016\)](#). We then passaged the initially generated pool of mutant viruses at low MOI to establish a genotype-phenotype linkage and select for functional HA variants. Deep sequencing of the variants before and after selection allowed us to infer the H3 site-specific amino-acid preferences. (B) The DMS experiments were completed in biological triplicate, starting from independent preps of the wildtype HA genes to create the mutant plasmids. In addition, we passaged and deep sequenced library 3 in technical replicate, denoted 3-1 and 3-2, to estimate the experimental noise within a single biological replicate. (C) Mutation frequencies of nonsynonymous, stop, and synonymous mutations for the mutant DNA, mutant virus, and unmutated DNA and virus controls. There is selection against nonsynonymous and stop codons in the mutant viruses. The percentages signify the frequency of stop codons remaining in the passaged mutant viruses relative to their starting frequency in the mutant plasmid libraries after correcting for the stop codon frequencies in the wildtype DNA and viruses. (D) The Pearson correlations for the amino-acid preferences between each pair of replicates are shown. The correlation between the technical replicates is comparable to that between biological replicates, indicating that the low MOI passage contributes to much of the experimental noise.

existing knowledge about HA's biochemistry. For instance, sites that form structurally important disulfide bridges (sites 52 & 277, 64 & 76, 97 & 139, 281 & 305, 14 & 137-HA2, 144-HA2 & 148-HA2) ([Waterfield et al., 1981](#)) possess high preference for cysteine. At residues involved in receptor binding, there are strong preferences for the amino acids at

Model	$\Delta\text{AIC}$	Log Likelihood	Stringency	$\omega$	$\bar{\omega}$	$\omega_\alpha$	$\omega_\beta$
ExpCM	0.0	-8439.33	2.44	0.91	—	—	—
Goldman-Yang M5	2166.06	-9516.36	—	—	0.36	0.30	0.84
ExpCM, averaged across sites	2504.18	-9691.42	0.68	0.32	—	—	—
Goldman-Yang M0	2607.92	-9738.29	—	0.31	—	—	—

**Table 1: The site-specific amino-acid preferences are informative for describing human H3N2 evolution in nature.** We implemented several codon substitution models for phylogenetic fitting of an alignment of human H3N2 HA sequences. The maximum likelihood values for each model were compared using the Akaike information criteria ( $\Delta\text{AIC}$ ) (Posada and Buckley, 2004). An experimentally-informed codon substitution model (ExpCM) built from the preferences averaged across all replicates performs better than conventional substitution models, specifically the M0 and M5 models in Yang et al. (2000). A non-site-specific ExpCM informed by preferences averaged across all sites performs comparably to the GY94 class of models, indicating the importance of site-specificity in the ExpCM. The optimized parameters for each model are also shown.

sites Y98, D190, W153, and S228. A positively charged amino acid at site 329 is important for cleavage activation of the HA0 precursor, and indeed this site exhibits a high preference for arginine (Kido et al., 1992; Stech et al., 2005). However, a notable exception occurs at the start codon at position -16, which does not show a strong preference for methionine. This codon is part of the signal peptide and is cleaved from the mature HA protein. One possible explanation for why we do not see a strong preference for Met at this site is due to alternative translation initiation occurring at a downstream or upstream start site, as has been described in [need to list citations here, inc. Girard 2011 *Infection Genetics and Evolution*, Chen 2001 *Nat Med*].

### The H3 stalk is relatively mutationally tolerant

We next sought to investigate the inherent mutational tolerance of the Perth/2009 HA. Figure 3 shows the mutational tolerance as calculated from the rescaled Perth/2009 H3 preferences and the rescaled WSN/1933 H1 preferences mapped onto the HA crystal structures. We found antigenic site C and the most distal portion of the globular head near antigenic site B in the Perth/2009 H3 to be tolerant of mutations. Interestingly, the H3 stalk including the shorter  $\alpha$ -helix (helix A) is relatively mutationally tolerant compared to the tolerance of the globular head domain. This observation suggests that the stalk may be prone to escape from antibodies, and agrees with previous work demonstrating that it is possible to select for antigenic mutants in H3 by broadly-neutralizing stalk-targeting antibodies (Ekiert et al., 2011; Friesen et al., 2014; Chai et al., 2016; Yamayoshi et al., 2017).

The sites inside the receptor binding pockets are highly functionally constrained and were found to be relatively mutationally intolerant in both H3 and H1 (Wilson et al., 1981). In contrast, the residues surrounding the receptor binding pocket are fairly mutationally

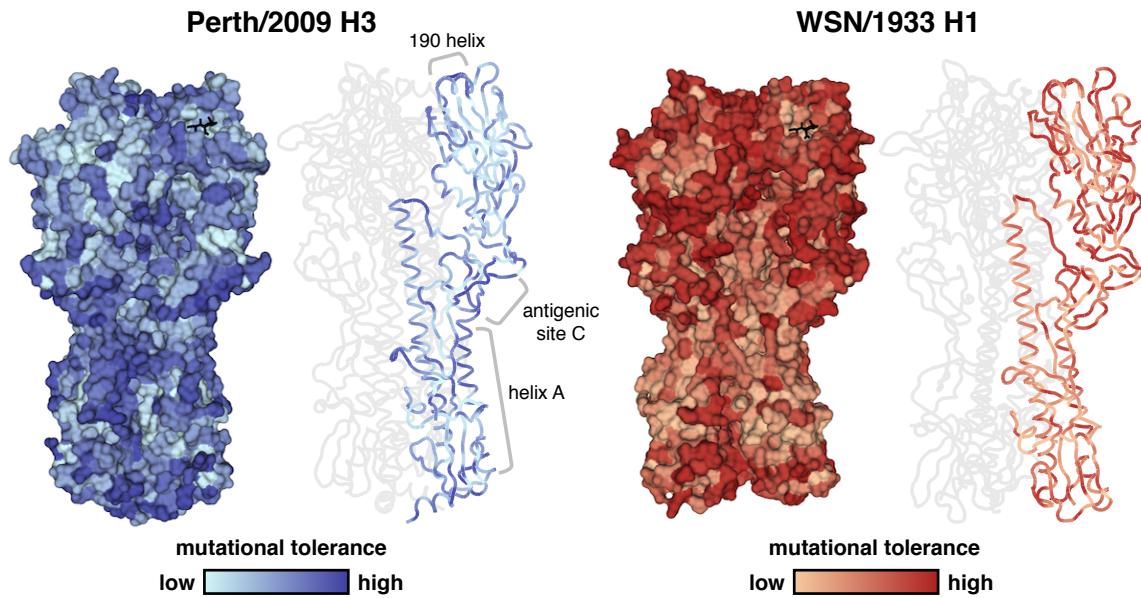


**Figure 2: The site-specific amino-acid preferences of the Perth/2009 HA.** This logplot shows the site-specific amino-acid preferences for the averaged replicates rescaled by the stringency parameter (Table 1) estimated by phydms. The height of each letter is proportional to its preference at that site, and the preferences for all sites are normalized to sum to 1. The sites are in H3 numbering. The top overlay bar shows the relative solvent accessibility. The bottom overlay bar is colored by the HA domain (sig. pep. = signal peptide, HA1 ecto. = HA1 ectodomain, HA2 ecto. = HA2 ectodomain, TM = transmembrane domain, cyto. tail. = cytoplasmic tail). The letters directly above each logo indicate the wildtype amino acid at that site.

tolerant, which may contribute to antigenic evolution as these sites are under strong immune pressure. (Wiley et al., 1981).

### The experimental measurements can help discriminate successful influenza virus lineages

Do the preferences reveal inherent differences between lineages of human H3N2 influenza virus that have succeeded and those that have died out? To examine this, we calculated the effect of trunk and side branch mutations from an H3N2 phylogeny, shown in Figure 4A, from 1968-2012 using our preference dataset. Strains with mutations that we measured to



**Figure 3: Mutational tolerance mapped onto each site of HA.** Mutational tolerance as calculated by the Shannon entropy of a given site's amino-acid preferences are mapped onto the structure of the H3 trimer (PDB 4O5N; [Lee et al. \(2014\)](#)) and the H1 trimer (PDB 1RVX; [Gamblin et al. \(2004\)](#)), with both trimers in approximately the same orientation. The site entropies were calculated from the preferences measured in the Perth/2009 H3 (left panel) from this study, or the preferences measured in the WSN/1933 H1 (right panel) from [Doud and Bloom \(2016\)](#). Lighter shades of blue or red signify low mutational tolerance, while darker shades of blue or red signify high mutational tolerance. For each HA, the structure on the left side colors the full HA trimer, while the structure on the right side colors only one of the monomers. The sialic acid receptor is shown as black sticks. The Perth/2009 H3 shows relatively high mutational tolerance in the stalk region compared to the head region. The head region of the WSN/1933 H1 is mutationally tolerant compared to the relatively intolerant stalk region.

be more beneficial to viral growth tend to succeed in nature. Figure 4B shows the median  $\log_2$  mutational effects and the interquartile ranges for trunk and side branch mutations in five-year windows for every year from 1968-2012. On average, trunk mutations tend to be towards more preferred amino acids compared to side branch mutations, and this was true for all windows. Next we calculated the effect of all trunk and side branch mutations from the entirety of the phylogenetic tree. We found that on average, trunk mutations are significantly more favorable than side branch mutations (Figure 4C). Because a number of tip nodes are likely from egg- or cell-passaged isolates and our experiments were performed in cell culture, we were curious to see if our measurements would lead to higher estimations of mutational effects for terminal nodes. We found that terminal node mutations are on average towards less preferred amino acids than internal node mutations, and that both internal and terminal node mutations are significantly less favorable than those

on the trunk. Therefore, strains that have accumulated mutations that we measured in our experiments to be unpreferred generally die out in nature, while more favored mutations provide a selective advantage to the surviving trunk. [*The average effect of trunk mutations near zero may also suggest that there is a balance between preferred and unpreferred mutations on the trunk.*] These findings demonstrate the importance of the functional impacts of HA mutations in determining lineage success.

Rather than estimating an average effect of mutations for trunk and side branch lineages, we can directly compare nodes in the tree by scoring the HA sequence of every node using the preferences to quantify a metric which we will refer to as *sequence preference*. Figure 5 shows that the sequence preference of trunk nodes tends to be higher than that of side branch nodes. Interestingly, the sequence preference of all nodes increases over time until it reaches the Perth/2009 node and its closely related nodes, which exhibit higher sequence preference than the trunk. The observation that the strain in which we performed our deep mutational scan has one of the highest sequence preferences illustrates epistatic interactions among mutations such that an unpreferred mutation in one background may be preferred in another. Yet, we are able to distinguish trunk mutations as more favorable than side branch mutations, indicating that the preferences are still of utility over short evolutionary timescales.

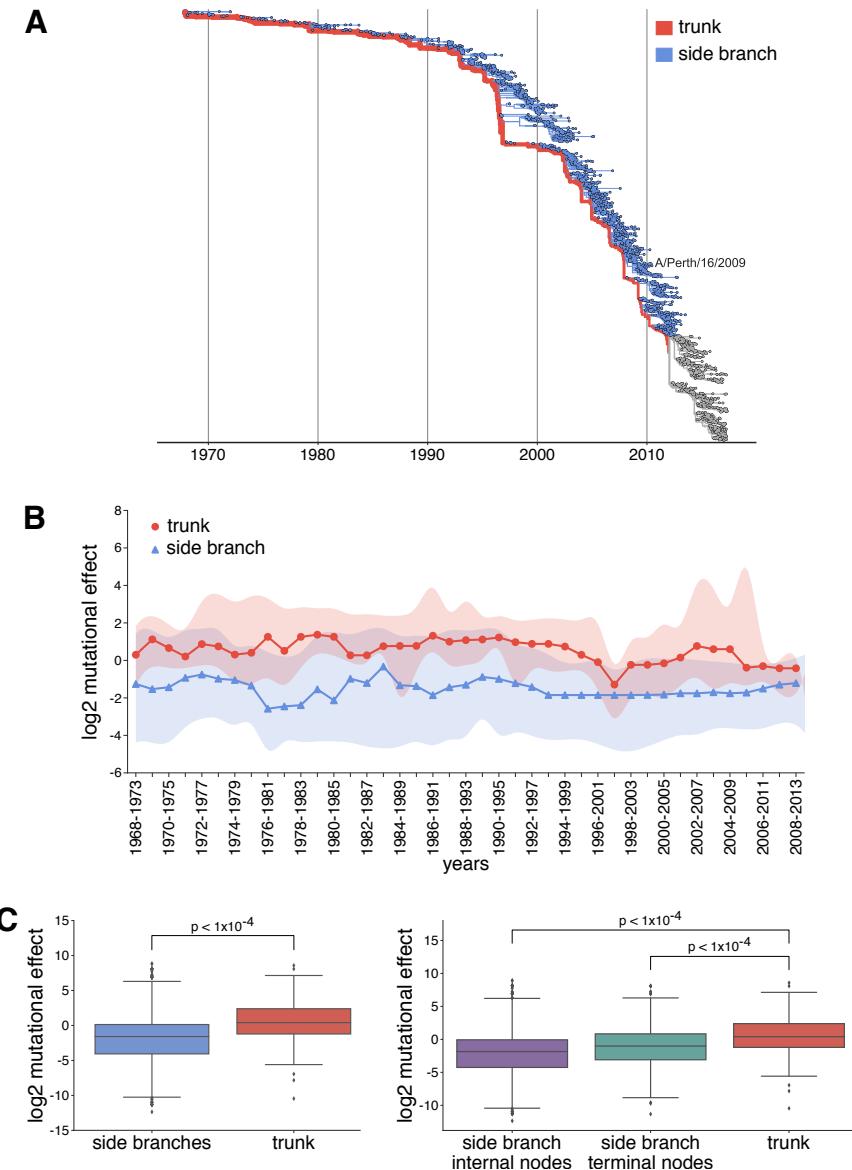
Can we then distinguish lineage-specific mutational effects using the preferences measured in a related HA homolog? Figure 6 shows the effect of H3 trunk and side branch mutations as quantified by the WSN/1933 H1 preferences from Doud and Bloom (2016). It is evident that we do not see trunk mutations significantly more favored than side branch mutations using these preferences, suggesting that our ability to discriminate successful and unsuccessful lineages degrades over sufficiently long evolutionary distances.

[*may add text here on using H1 preferences for H1N1 tree*]

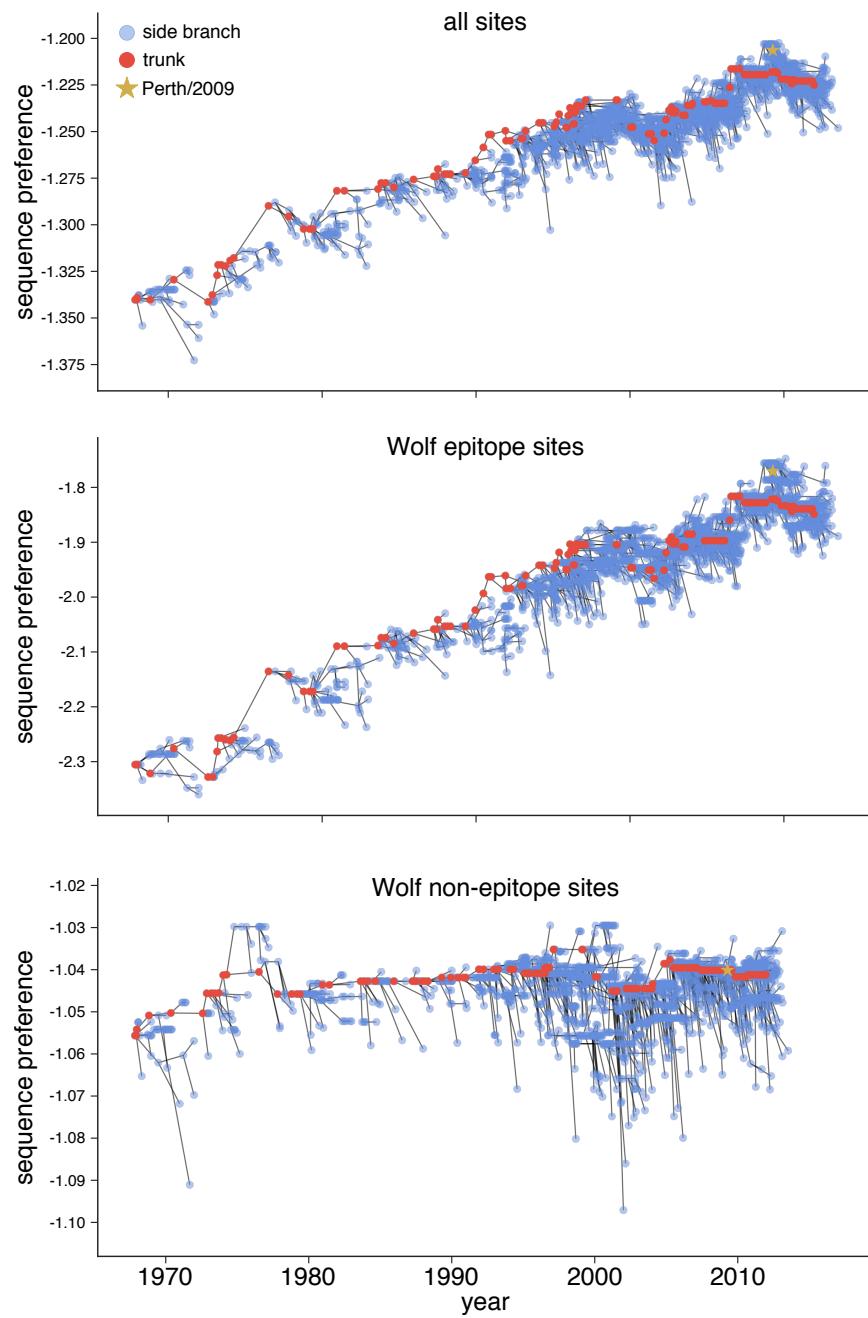
## Comparing H1 and H3 preferences

## DISCUSSION

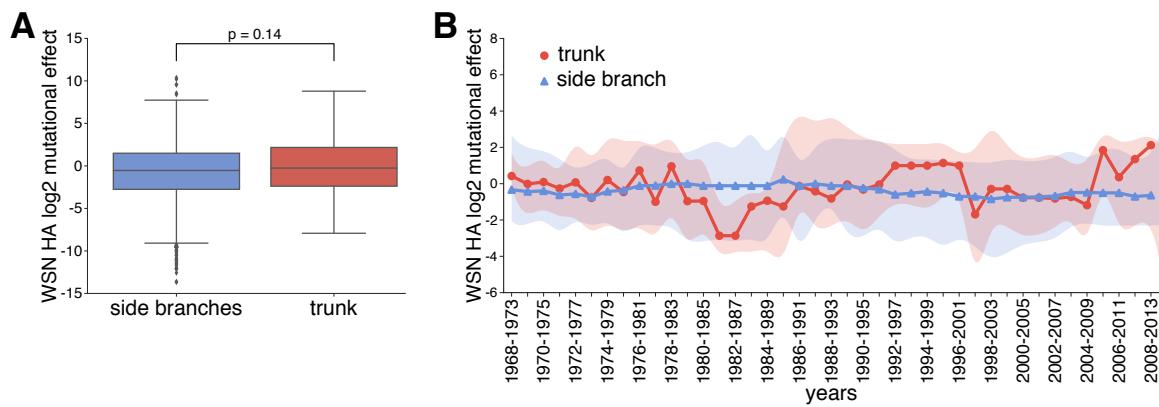
We have measured the effect of all possible single amino-acid mutations to Perth/2009 H3 on viral growth in cell culture.



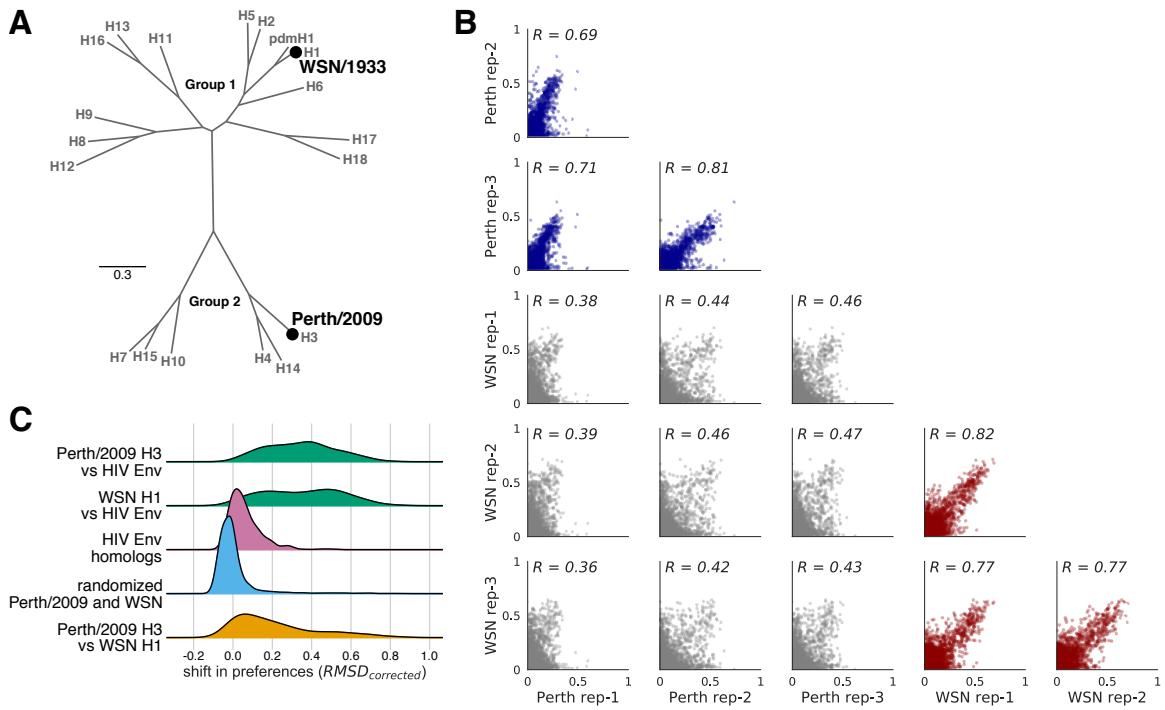
**Figure 4: Mutations in evolutionary successful strains tend to be more favorable than in strains that die out.** (A) Phylogenetic tree of human H3N2 influenza virus from 1968-present. The trunk is shown in red, and side branches are shown in blue. The gray branches represent the part of the tree for which we cannot yet distinguish the trunk from side branches. (B) Using the Perth/2009 H3 preferences, we calculated the  $\log_2$  mutational effect for trunk and side branch mutations in windows of 5 years for every year from 1968-2013. The median  $\log_2$  mutational effect in a given window is shown as circles for trunk mutations and triangles for side branch mutations. The shaded region demarcates the interquartile range of trunk and side branch mutational effects. The median trunk mutational effects are consistently higher than the median side branch mutational effects for all windows. (C) The  $\log_2$  mutational effect for all side branch and all trunk mutations (left panel), in addition to all mutations in internal nodes and terminal nodes on the side branches (right panel) are shown. The preferences were randomized for a number of iterations to estimate significance. The effects of trunk mutations are higher than side branch, internal and terminal node, mutations.



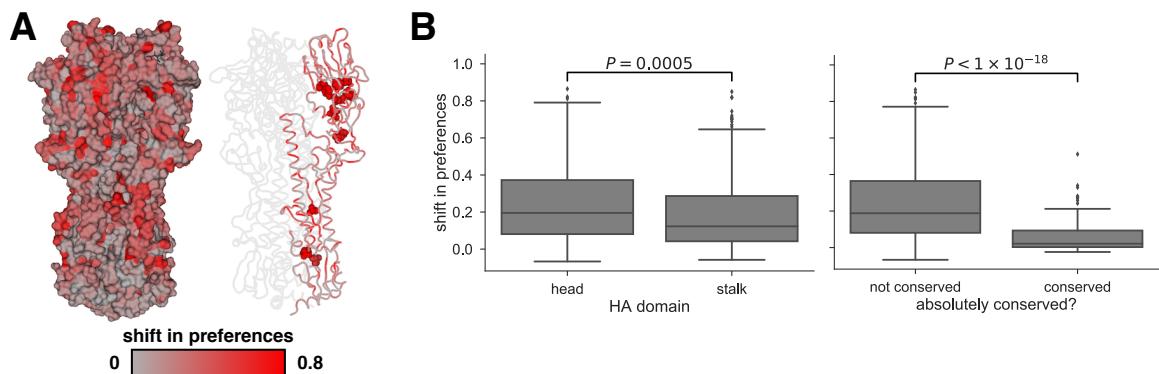
**Figure 5: The trunk exhibits higher sequence preference than side branches.** figure text



**Figure 6: The WSN/1933 H1 preferences do not reveal differences in trunk vs side branch mutational effects** (A) We calculated the log<sub>2</sub> mutational effects of the same set of trunk and side branch mutations from the inferred H3N2 phylogeny in Figure 4 using the WSN/1933 H1 preferences. There is not a significant difference in trunk vs side branch mutational effects. (B) We also performed the same sliding window analysis shown in Figure 4B, but using the WSN/1933 H1 preferences. There is not a distinct difference in trunk and side branch mutational effects.



**Figure 7: The HA homologs exhibit many large shifts in preference compared to shifts for other viral protein homologs** (A) A phylogenetic tree of the HA subtypes, with the two HA's, WSN/1933 H1 and Perth/2009 H3, for which we have measured amino-acid preferences denoted on the tree. The WSN/1933 H1 and the Perth/2009 H3 share  $\sim 42\%$  amino-acid identity. (B) The correlations of the amino-acid preferences for replicates both within and between the two HA homologs. The within-Perth/2009 and the within-WSN/1933 correlations are shown in blue and red, respectively. The between homolog correlations are in gray. The correlations for replicates within a homolog are higher than for replicates between homologs. (C) The distribution of shifts in preference for various homolog pairs are shown.



**Figure 8: Shifts in preferences mapped onto the structure of HA** (A) The preference shifts as calculated by  $RMSE_{corrected}$  between the two HA homologs is mapped onto the structure of HA (PDB 4O5N, citation). The left structure shows the HA trimer, and the right structure colors one of the monomers. The sialic acid receptor is shown in black sticks. Gray indicates little shifts in preference, while red indicates large shifts in preference. The top ten most shifted sites are shown in spheres on the monomer. (B)

## METHODS

### HA numbering

Unless otherwise indicated, all sites are in H3 numbering, with the signal peptide in negative numbers, the HA1 subunit in plain numbers, and the HA2 subunit denoted with "(HA2)". The conversion between sequential numbering of the A/Perth/16/2009 HA and H3 numbering was performed using an HA numbering Python script (available at [https://github.com/jbloomlab/HA\\_numbering](https://github.com/jbloomlab/HA_numbering)).

### Creation of MDCK-SIAT1-TMPRSS2 cell line

The human TMPRSS2 cDNA ORF was ordered from OriGene (NM\_005656), PCR amplified, and cloned into a pHAGE2 lentiviral vector under an EF1 $\alpha$ -Int promoter and attached to mCherry through an IRES...etc etc [Need to look at Katie's notebooks for this...]

### Generation of HA codon mutant plasmid libraries

Recombinant A/Perth/16/2009 (HA, NA)  $\times$  A/Puerto Rico/8/1934 influenza virus, NIB-64, NR-41803 was ordered from BEI Resources, NIAID, NIH. Bulk RNA from the viral sample was extracted using the QIAamp Viral RNA Mini Kit (QIAGEN) according to manufacturer's instructions. The Perth/2009 HA and NA genes were then reverse transcribed, PCR amplified, and cloned into the pHW2000 (Hoffmann et al., 2000) and pICR2 [cite?] plasmid backbones.

The codon-mutant libraries were generated using a PCR-based approach described in Dingens et al. (2017).

### Generation and passaging of mutant viruses

The mutant virus libraries were generated and passaged using the approach described in Doud and Bloom (2016) with several modifications.

### Barcoded subamplicon sequencing

### Analysis of deep sequencing data

### Inference of phylogenetic trees

[We downloaded X sequences from the Influenza Virus Resource ?.... etc. inferred the tree, ancestral state reconstruction, visualized the tree. Mark Perth/2009 on the tree] To parse out trunk mutations from side branch mutations, we first defined a set of recent nodes sampled on or after Jan. 1, 2017, and traced these nodes back to their most recent common ancestor.

## Quantification of mutational effects and sequence preferences from an H3N2 phylogeny

### Data availability and source code

Deep sequencing data are available from the Sequence Read Archive under BioSample accessions SAMN08102609 and SAMN08102610. Computer code used to analyze the data and produce the results in the paper are in...

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