Evaluating H5N1 Vaccine Breadth using Computationally-Designed Proteins

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Abstract

The ongoing outbreaks of highly pathogenic avian influenza H5N1 viruses, particularly genotype D1.1 within clade 2.3.4.4b, and their increasing spillover into mammalian hosts underscore a growing pandemic threat. Although prior exposure to seasonal influenza viruses may provide limited cross-reactive immunity, H5N1 viruses are antigenically distinct. Moreover, existing H5N1 vaccine stockpiles were developed against earlier viral clades and may provide limited protection against currently circulating strains. To address this challenge, we designed a set of 22 antigenically diverse hemagglutinin (HA) proteins intended to evaluate the breadth and durability of protection elicited by candidate H5N1 vaccines. We show that deep learning models can forecast close to 80% of mutations that have occurred in clade 2.3.4.4b. Consistent with these predictions, several designed HA variants exhibited escape from antibodies elicited by D1.1 vaccination as well as from broadly neutralizing monoclonal antibodies, highlighting potential pathways for future antigenic evolution. Together, these results establish a framework for proactively assessing vaccine robustness against both circulating and anticipated H5N1 variants.

1. Introduction

A major challenge in mitigating viral outbreaks is anticipating how viral pathogens will evolve under immune pressure. Vaccines and antiviral therapeutics are typically evaluated against currently circulating or historically observed strains, yet rapidly evolving RNA viruses can acquire mutations that reduce vaccine-induced protection. This challenge is particularly acute for highly pathogenic avian influenza viruses such as H5N1, which continue to circulate globally across animal reservoirs and pose an ongoing zoonotic and pandemic threat (Kok et al., 2025). Although current vaccine evaluation frameworks assess breadth against known viral diversity, they provide limited ability to prospectively evaluate protection against variants that have not yet emerged.

Influenza hemagglutinin (HA) protein represents an especially challenging target for vaccine design because immune pressure continuously drives antigenic evolution (Choi et al., 2024). Mutations within HA antigenic sites can substantially alter antibody recognition while preserving viral fitness(Dadonaite et al., 2024). Traditional experimental approaches for assessing escape potential, including serial viral passaging or deep mutational scanning, are resource intensive and often limited to specific antibodies and viral genotypes (Kikawa et al., 2025a; Loes et al., 2024; Kikawa et al., 2025b). A scalable framework capable of prospectively modeling plausible future HA evolution and directly evaluating vaccine robustness against these trajectories would substantially strengthen pandemic preparedness efforts.

Recent advances in computational evolutionary modeling have created new opportunities to forecast viral evolution directly from sequence data. Alignment-based generative models and protein language models (PLMs) trained on natural protein sequences can capture evolutionary constraints governing viral fitness and immune escape (Mehrotra et al., 2025). These models have demonstrated the ability to identify mutations enriched during viral evolution and predict functional consequences of sequence variation across diverse viral families (Gurev et al., 2025). Here we focus on H5N1 Avian influenza to show how computationally predicted evolutionary trajectories can be translated into practical experimental tools for vaccine evaluation (Fig. 1a).

We first show that both alignment-based models and protein language models can successfully capture observed mutations in avian influenza HA, and to a much higher degree than experimental deep mutational scans. We then compu-

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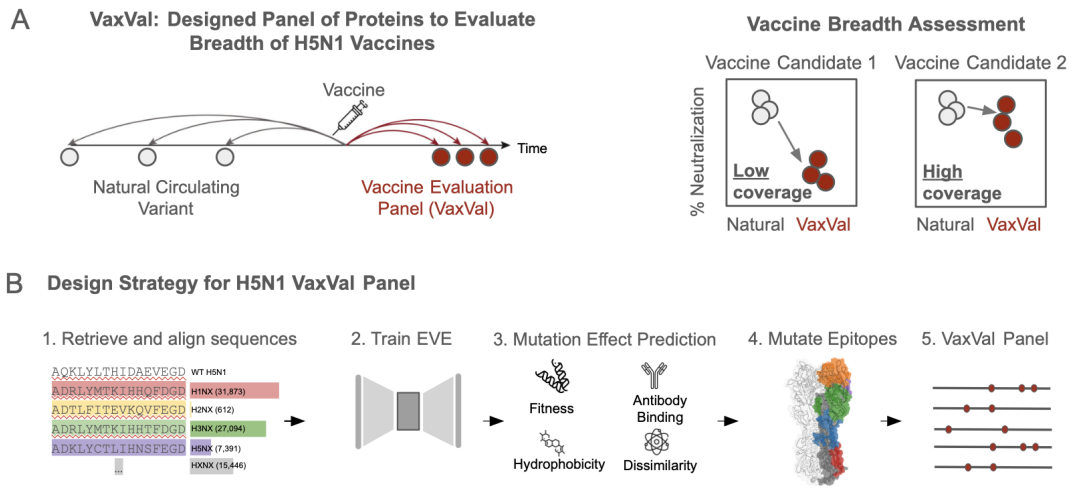


Figure 1. **Designed panel of H5 hemagglutinin proteins for vaccine evaluation.** A) Current vaccine evaluation approaches assess protection against circulating or past variants, which poorly predict efficacy against future viral evolution. To overcome this, we developed VaxVal, a computationally designed panel of H5 hemagglutinin variants that serves as a proxy for likely future evolutionary trajectories, enabling proactive vaccine evaluation. B) VaxVal panel design for H5N1 genotype D1.1. We trained the deep learning model EVE on historical hemagglutinin sequences predating the emergence of the D1.1 strain. We selected candidate mutations by integrating multiple constraints: high predicted fitness scores, known antibody binding, hydrophobicity, and dissimilarity between the mutant and wildtype amino acid. The final panel comprises 22 variants representing plausible future mutations.

tationally design VaxVal, a panel of 22 H5 hemagglutinin variants that include evolutionarily plausible combinations of mutations (Fig. 1b). Using the VaxVal panel, we evaluated the breadth of antibody responses elicited by an H5N1 vaccine candidate (D1.1). We identify multiple designed variants exhibiting reduced recognition by vaccine-induced antibody responses, demonstrating that computationally generated proteins can reveal potential escape pathways before their emergence in circulating viruses. By making this experimentally validated HA panel publicly available, we establish a scalable platform for prospective vaccine stress-testing that complements traditional surveillance and antigenic characterization approaches. More broadly, this framework provides a path toward evaluating vaccine resilience against future viral evolution rather than solely against past and present diversity.

2. Results

2.1. Computational models forecast H5N1 evolution

The extent to which computational models can be used to predict future variants for early vaccine evaluation, depends on their ability to predict how a virus will evolve. We first assessed whether computational models can predict the evolution of a recent H5N1 clade. We used descendants of the recent D1.1 (A/American Wigeon/South Carolina/22-000345- 001/2021) strain (emerged in November 2021) as the test set (Fig. S1). We evaluated six models: (i) the alignment-based model EVE (Frazer et al., 2021); (ii) Ensemble ESM-1v, a sequence-only protein language model (PLM) (Meier et al., 2021); (iii) Tranception, a sequence-only PLM trained on UniRef100 (Notin et al., 2022); (iv) two versions of SaProt, a structure-aware PLM trained on either AlphaFoldDB (SaProt-AF) or further refined using structures from the Protein Data Bank (SaProt-PDB) (Su et al., 2023); and (v) Tranception with MSA retrieval (Notin et al., 2022) and (vi) VESPA (Marquet et al., 2022).

First, we assessed the ability to recall mutations that were frequently observed during D1.1 evolution (Fig. 2a). Because the vast majority of available influenza sequences are derived from the H1 and H3 subtypes (Fig. 1a), we evaluated EVE models trained on datasets with different subtype specificities. We found that EVE robustly recalled the most frequently occurring mutations regardless of the training dataset used (Fig. S2). EVE correctly identified all mutations observed more than 1000 times in decendent D1.1 strains. In contrast, protein language models (PLMs) showed substantially more variable performance (Fig. 2a). Among PLMs, Tranception outperformed SaProt-AF, SaProt-PDB, ESM-1v, and the hybrid model VESPA. Incorporating H5N1-specific multiple sequence alignment retrieval into Tranception further improved performance, yielding recall comparable to EVE.

Both EVE and Tranception with retrieval captured approximately 80% of all mutations observed during the subsequent two years of D1.1 evolution. We note that perfect recall is not expected, as some mutations may arise under weak or near-neutral selection.

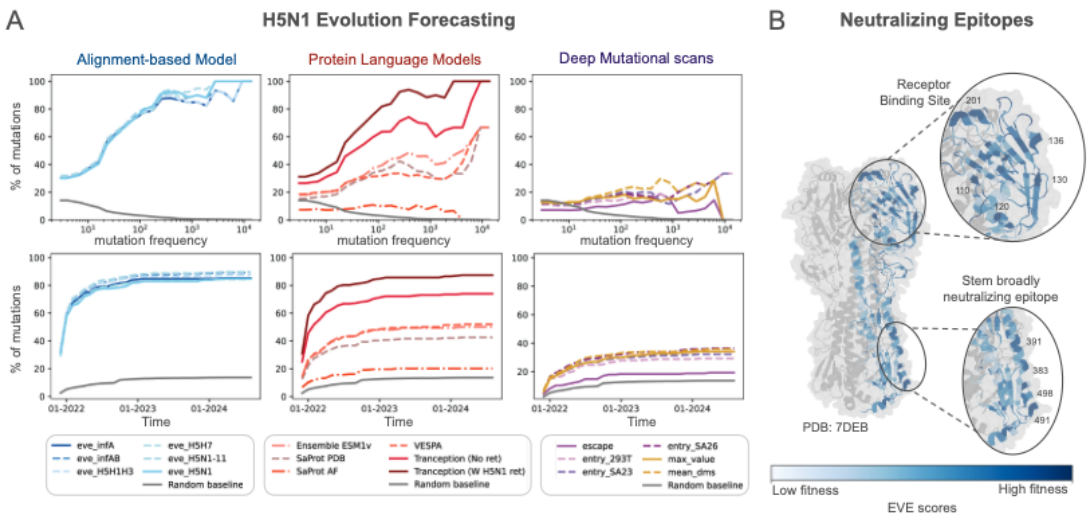


Figure 2. **Computational models forecast H5N1 hemagglutinin evolution.** A) Performance of alignment-based models, protein language models, and deep mutational scans (DMS) in predicting the most frequently observed mutations (top) and recalling mutations that arose following the emergence of the H5N1 D1.1 lineage (bottom). The alignment-based model EVE and the hybrid model Tranception with retrieval (which integrates a protein language model with alignment-derived evolutionary information) achieve the highest performance across both tasks. DMS experiments measured distinct phenotypic components of viral fitness (e.g., escape from sera, entry into different cell lines). DMS scores were also aggregated per mutation using either a max or mean operator across all measured phenotypes. B) EVE scores are enriched in antigenically relevant regions proximal to the receptor-binding domain, as well as within a stem epitope targeted by broadly neutralizing antibodies.

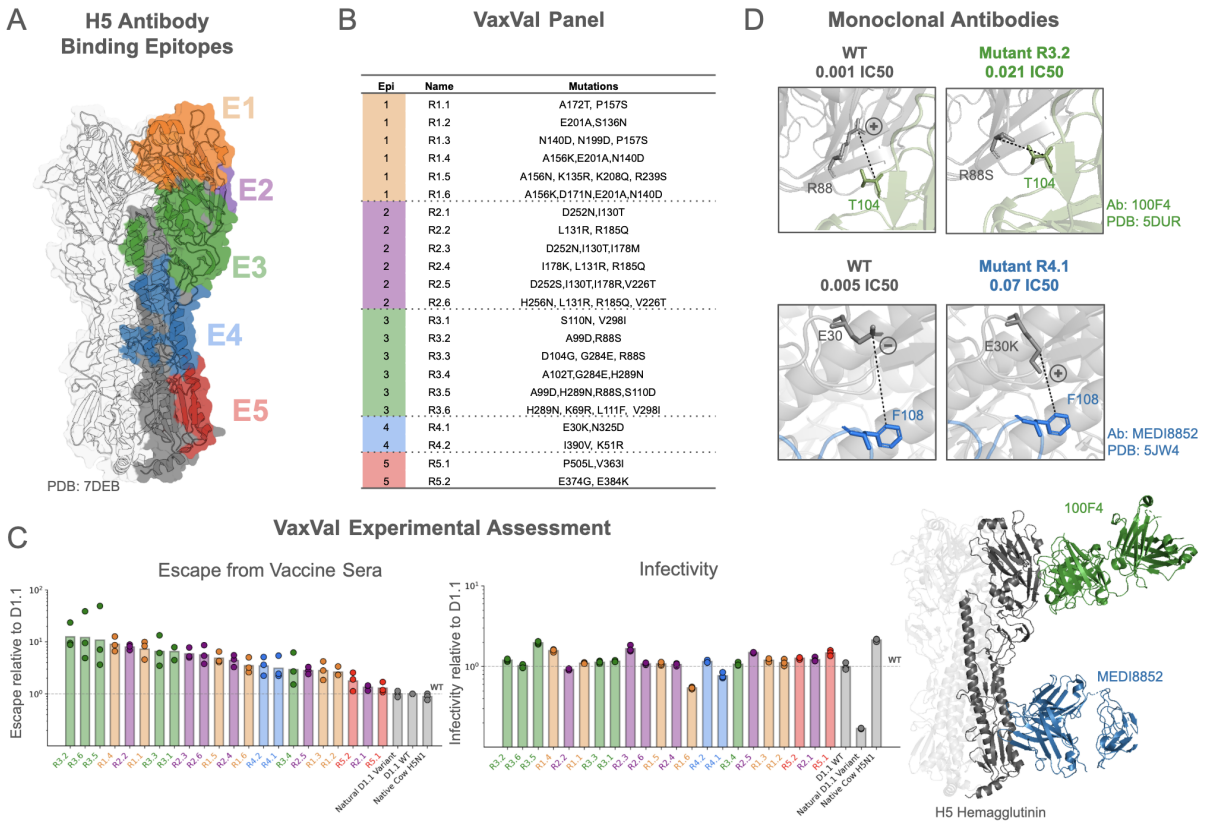
In contrast to computational models, deep mutational scanning (DMS) experiments measuring individual phenotypes recalled fewer than 40% of mutations observed during the evolution of the D1.1 lineage. The DMS datasets quantified four phenotypic readouts: escape from polyclonal sera and viral entry across three distinct cell lines. Each assay captured only a subset of observed evolutionary variation, suggesting that no single phenotype is sufficient to explain viral evolutionary dynamics. Because viral fitness likely reflects a composite of multiple selective pressures, we evaluated whether aggregating measurements across assays improved predictive performance. Specifically, we computed per-mutation maximum and mean scores across all phenotypic readouts. However, neither aggregation strategy substantially improved recall (Fig. 2a).

Given these results, we selected EVE trained on all Influenza A hemagglutinin sequences for VaxVal panel design. As shown in Fig. 2b, EVE scores highlight known antigenically relevant regions across the protein, particularly epitopes proximal to the receptor-binding domain and within the conserved stalk domain.

2.2. VaxVal Panel Identifies Escape from D1.1 Vaccination

In order to design a comprehensive evaluation panel for vaccine protection breadth, we must identify prominent antigenic regions on the hemagglutinin protein. To do this, we identified all known antibody-binding footprints (defined as residues within 5 Å of antibody atoms) for influenza A hemagglutinin. We then spatially clustered all antibody-contacting residues into five epitopes (Fig. 3a). Three epitopes were located in the HA head region (E1–E3) and two in the stem region (E4–E5). We used EVE, trained on all influenza A hemagglutinin sequences, to generate all possible double, triple, and quadruple mutants within each epitope. Mutants were ranked using a composite score incorporating predicted evolutionary score, hydrophobicity and charge dissimilarity relative to wild-type residues, and solvent accessibility as a proxy for antibody exposure. Designs containing mutations within 10 Å of one another were excluded to avoid spatially clustered substitutions. A final set of 22 VaxVal constructs was selected based on this ranking (Fig. 3b), representing variants with high-escape potential.

We used the VaxVal panel to evaluate the breadth of protection elicited by D1.1 immunization. Three rabbits were immunized with D1.1, receiving a primary immunization followed by a booster dose after two weeks; sera were collected one week after boosting. Infectivity and neutralization susceptibility were measured using H5N1 pseudovirus-based assay Fig. 3c for the VaxVal panel along with two naturally circulating variants: A/dairy cow/New Mexico/A240920343-90/2024 (GISAID ID 19091701; hereafter "Native Cow") and a low-frequency D1.1 variant identified from an infected adolescent ("BC Teen V1"). Both



Epi	Name	Mutations
1	R1.1	A172T, P157S
1	R1.2	E201A,S136N
1	R1.3	N140D, N199D, P157S
1	R1.4	A156K,E201A,N140D
1	R1.5	A156N, K135R, K208Q, R239S
1	R1.6	A156K,D171N,E201A,N140D
2	R2.1	D252N,I130T
2	R2.2	L131R, R185Q
2	R2.3	D252N,I130T,I178M
2	R2.4	I178K, L131R, R185Q
2	R2.5	D252S,I130T,I178R,V226T
2	R2.6	H256N, L131R, R185Q, V226T
3	R3.1	S110N, V298I
3	R3.2	A99D,R88S
3	R3.3	D104G, G284E, R88S
3	R3.4	A102T,G284E,H289N
3	R3.5	A99D,H289N,R88S,S110D
3	R3.6	H289N, K69R, L111F, V298I
4	R4.1	E30K,N325D
4	R4.2	I390V, K51R
5	R5.1	P505L,V363I
5	R5.2	E374G, E384K

Figure 3. **VaxVal panel identifies antibody escape mutations in H5 hemagglutinin.** A) Defining antibody-binding epitopes across H5 hemagglutinin (HA). Antibody contact residues (5A) were identified from structures in the PDB and grouped by spatial clustering into five distinct epitopes. B) Design of the VaxVal panel. Twenty-two HA variants were generated, each incorporating mutations within a one of the five defined epitopes. C) VaxVal variants exhibit escape from vaccine-induced polyclonal sera. All variants show reduced neutralization by sera from immunized rabbits relative to the wild-type, vaccine-matched D1.1 strain (dotted line). Variants with the greatest escape predominantly include mutations in epitopes E1, E2 and E3. Many high-escape variants retain infectivity, indicating preservation of functional viral entry (right panel). D) VaxVal panel constructs escape broadly neutralizing H5 monoclonal antibodies more than WT D1.1. Full panel results in Fig. S4.

naturally occurring variants exhibited similar levels of escape comparable to the D1.1 wild-type strain, despite the Native Cow variant have 10 mutations (see Table S1). Interestingly, some VaxVal constructs were mutated at the same positions as naturally occurring variants (131, 156, 226), though the mutations selected for VaxVal are predicted to have stronger impact on antibody binding.

The VaxVal panel revealed substantial resistance to neutralization by D1.1-induced sera, even though constructs contained only 2–4 mutations. In particular, designed variants R3.2 R3.5, R3.5, R1.4, and R2.2 exhibited approximately 10-fold reductions in neutralization relative to wild-type D1.1. The most resistant constructs predominantly carried mutations in the HA head domain, consistent with its known immunodominance relative to the more conserved stalk region (Zost et al., 2019). Despite their escape from vaccine-induced sera, all constructs were infectious, with many showing infectivity levels comparable to or exceeding that of wild-type D1.1 (Fig. 3c). We observed no relationship between the number of mutations and fold change in neutralization or infectivity (Fig. S3).

To further characterize the effects of mutations in the VaxVal variants, we measured neutralization using two broadly neutralizing monoclonal antibodies (mAbs), MEDI8852 and 100F4, targeting the stem and head of the protein, respectively (Fig. 3a, S4). In Fig. 3d, we highlight two high-escape variants, each originating from a distinct epitope group, and show how mutations alter binding to their corresponding cognate mAbs. Together, these results demonstrate VaxVal's ability to both generate plausible evolutionary variants and systematically assess potential immune escape across antigenically distinct regions of the protein. While our panel is designed around H5N1, we anticipate that the workflow can be scaled across pandemic threat viruses.

2.3. Impact Statement

This work introduces a computational framework for proactively evaluating vaccine robustness against future viral evolution. Using evolutionary deep learning models, we generate biologically plausible H5N1 hemagglutinin variants that represent potential future evolutionary trajectories and demonstrate that these models recover the majority of mutations that subsequently emerged during H5N1 evolution. Experimental validation shows that several designed variants escape vaccine-induced antibody responses while retaining functionality, revealing potential vulnerabilities before such variants are observed in nature. More broadly, this study highlights how generative biological models can move beyond prediction to guide experimental design, providing a scalable approach for prospective vaccine stress-testing that can be extended to other rapidly evolving pathogens and support future pandemic preparedness efforts.

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A. Methods

A.1. Alignment-based models

We assessed the performance of 3 alignment-based models: a position-specific scoring matrix (PSSM), potts model (EVmutation), and variational autoencoder (EVE).

A.1.1. EVE

To predict the effects of mutations capturing high-order dependencies between positions, we used EVE, a Bayesian Variational Autoencoder, as implemented in (Frazer et al., 2021). The architecture consists of a symmetric encoder and decoder architecture, each with 3 layers with 2,000-1,000-300 and 300-1,000-2,000 units respectively, as well as a 50-dimensional latent space. As generative models, VAEs can learn a complex distribution of the high-dimensional data on which they are trained, in our case, sequences from a specific protein family. We use single EVE models, rather than an ensemble of independent models as was reported in (Thadani et al., 2023a). Note, that we use the negative of the evolutionary index reported by the model.

A.2. Protein language models

A.2.1. Tranception

Tranception (Notin et al., 2022) combines an autoregressive protein language model with inference-time retrieval from a MSA. We used Tranception Large (700M parameters) trained on UniRef100 using only the autoregressive inference without MSA retrieval as implemented in ProteinGym (Notin et al., 2023). Tranception averages the log ratios from both left-to-right and right-to-left scoring.

A.2.2. ESM-1v

ESM-1v (Meier et al., 2021) has a Transformer encoder architecture similar to BERT [Devlin et al., 2019] and was trained with a Masked-Language Modeling (MLM) objective on UniRef90. We use the implementation presented in ProteinGym (Notin et al., 2023) to handle sequences that are longer than the model context window (i.e., 1023 amino acids), and ensemble across 5 models.

A.2.3. SaProt

SaProt (Su et al., 2023) introduces a structure-aware vocabulary, into protein language modeling by training on Foldseek (van Kempen et al., 2022) 3Di tokens which represent the local geometric conformation information of each residue relative to its spatial neighbors. These 3Di tokens are combined with typical amino acid residue tokens as input to the SaProt model, which utilizes an ESM-2 Transformer architecture (Lin et al., 2023) but expands the embedding layer to encompasses 441 structurally-aware tokens instead of the original 20 amino acid residue tokens. We use (1) SaProt-650M-AF2, trained on approximately 40 million AF2 sequences/structures (from UniRef50) which notably explicitly excludes all viral proteins though implicitly included hundreds of thousands of prophages; and (2)SaProt-650M-PDB, which continuously pre-trains the SaProt-650M-AF2 model on the PDB.

For structure inputs to Foldseek calculation, we folded monomeric forms of H5N1 D1.1 Hemagglutinin with AlphaFold3.

A.3. Mutation effect scoring

Generative models learn from the distribution of protein sequences collected as a result of billions of evolutionary experiments to capture the biochemical and structural constraints governing functional proteins. These models are trained to learn the distribution of natural, functional sequences.

For a given protein x composed of residues $(x_1, x_2, ..., x_L)$ the relative fitness of mutated protein compared to its wild-type can be calculated in the following ways depending on the modeling objective.

The fitness of a mutant sequence x^{mutant} is calculated as:

$$\log \frac{P(x^{\text{mutant}})}{P(x^{\text{wildtype}})}$$

For ProGen2(Notin et al., 2022), an autoregressive model, the likelihood of x factorizes via the chain rule and is calculated as:

$$P(x) = \prod_{i=1}^{L} P(x_i|x_{<i})$$

For Tranception, an autoregressive model with bidirectional scoring:

$$P(x) = \frac{1}{2}\Big[\prod_{i=1}^{L} P(x_i|x_{<i}) + \prod_{i=1}^{L} P(x_i|x_{>i})\Big]$$

In the masked language model setting, for ESM-1v (Meier et al., 2021) and SaProt (Su et al., 2023), we use the masked marginal scoring function instead:

$$\sum_{i \in M} \log \frac{P(x_i = x_i^{\text{mutant}} \mid x_{-M})}{P(x_i = x_i^{\text{wildtype}} \mid x_{-M})}$$

where x_{-M} is the sequence x with masked residues at all mutated position M. Since we only consider single amino acid substitutions in this work, M contains only a single position.

For a VAE, as in EVE(Frazer et al., 2021), where the exact computation of log likelihood of a sequence is intractable, we approximate it with the Evidence Lower Bound (ELBO) used to optimize the VAE:

$$\log \frac{P(x^{\text{mutant}})}{P(x^{\text{wildtype}})} \approx ELBO(x^{\text{mutant}}) - ELBO(x^{\text{wildtype}})$$

The ELBO term itself is estimated via Monte Carlo sampling, using 20k samples from the approximate posterior distribution. These approximations have been shown to provide strong results in practice(Frazer et al., 2021). Note that this is the negative of the evolutionary index score outputted by the EVE model.

A.4. Forecasting D1.1 Evolution

To assess model performance in forecasting D1.1, we used the Global Initiative on Sharing All Influenza Data (GISAID) to identify all mutations arising from D1.1. Briefly, we took all hemagglutinin sequences deposited after November 2021 (the emergence date of D1.1 WT) and filtered for H5N1 sequences with a mutation distance up to 5 from the WT and used MUSCLE (Edgar, 2004) to generate an alignment, from which we calculated the frequency of all mutations.

A.5. Building Alignments for EVE

We used GISAID to build alignments of Influenza hemagglutinin sequences prior to November of 2021. Sequences were deduplicated and aligned to the D1.1 WT.

A.6. Deep mutational scanning assays

All deep mutational scanning assays were taken from (Dadonaite et al., 2024) which briefly, uses a lentivirus-based DMS assay to measure phenotypic effects of all single mutations on the D1.1 strain, which include cell entry, sialic acid receptor preference, HA stability, and serum neutralization (using sera from vaccinated mice or sera from vaccinated or infected ferrets).

A.7. Designing VaxVal Panel

To design the VaxVal panel, we used the Structural Antibody Database (SAbDab) (Dunbar et al., 2014) to scrape all PDBs with an antibody bound to an Influenza A hemagglutinin protein, giving a total of 210 structures. The antibody footprints of hemagglutinin were considered those within 5 Angstroms of an antibody residue, and were mapped onto the 7DEB PDB

structure of an H5 protein using SIFTS (Dana et al., 2019). Positions were filtered on the condition of interacting with atleast 3 unique antibodies. The final set of sites were converted into 3D coordinates and clustered into 5 distinct epitopes by agglomerative clustering (Pedregosa et al., 2011).

To design constructs per epitope, the top scoring mutations were selected by the following percentiles– 3% for epitope 4, 1% for epitope 1, 1% for epitope 3, 5% for epitope 5, and 1.5% for epitope 2. From this, all sets of double, triple, and quadruple mutations were generated as a plausible construct. Because the head of hemagglutinin is more antigenic, proteins with mutations in the head were prioritized–giving 6 proteins mutated in Epitope 1, 6 mutated in Epitope 2, 6 mutated in Epitope 3, 2 mutated in Epitope 4, and 2 mutated in Epitope 5 (Fig. 3a). Constructs were prioritized based on a composite score that sums the minimum scores assigned to any mutation in the construct. The scores correspond to 4 metrics: percentile in the epitope's fitness distribution, distance to any other mutating residue, surface accessibility, and residue dissimilarity. The latter two are taken from the EVEscape framework developed by Thadani et al. (2023b). Constructs with the highest scores were selected.

A.8. Recombinant DNA sequence plasmid design

All recombinant DNA work follows a previous protocol as outlined by Youssef et al. (2025).

A.9. Infectivity and neutralization assays

HIV pseudotyped H5N1 influenza virus-like particles were made by transfecting HEK-293T cells with plasmids encoding the luciferase reporter, the HIV gag-pol, the cow derived N1 neuraminidase, and the specific H5 hemagglutinin, in a 5:4:1:0.25 ratio (total 2,250 ng of DNA). Sera and monoclonal antibody neutralization also followed the procedure in (Youssef et al., 2025).

A.10. Rabbit immunization

Rabbit immunization experiments for D1.1 was conducted by Genscript (https://www.genscript.com). Three New Zealand white rabbits were used and given a 200 microgram mRNA vaccine followed by a booster shot two weeks later. Sera was collected one week following the booster shot.

B. Supplementary Figures

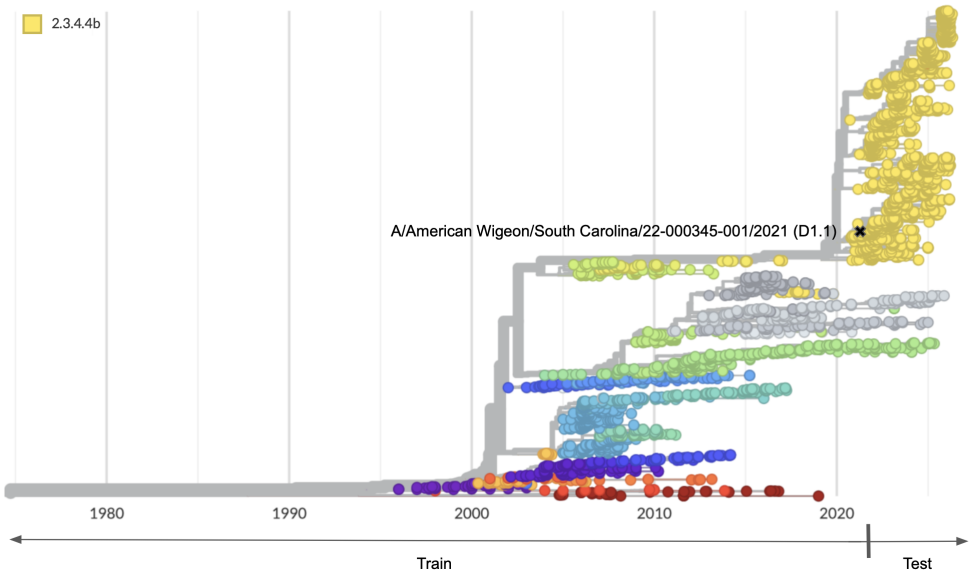

Figure 1. Phylogenetic tree of H5N1 hemagglutinin sequences deposited on Global Initiative on Sharing Avian Influenza Data(GISAID). Tree was made with NextStrain (Hadfield et al., 2018). EVE was trained on sequences prior to the emergence of D1.1 in clade 2.3.4.4b and tested for recapitulating mutations that appeared later.

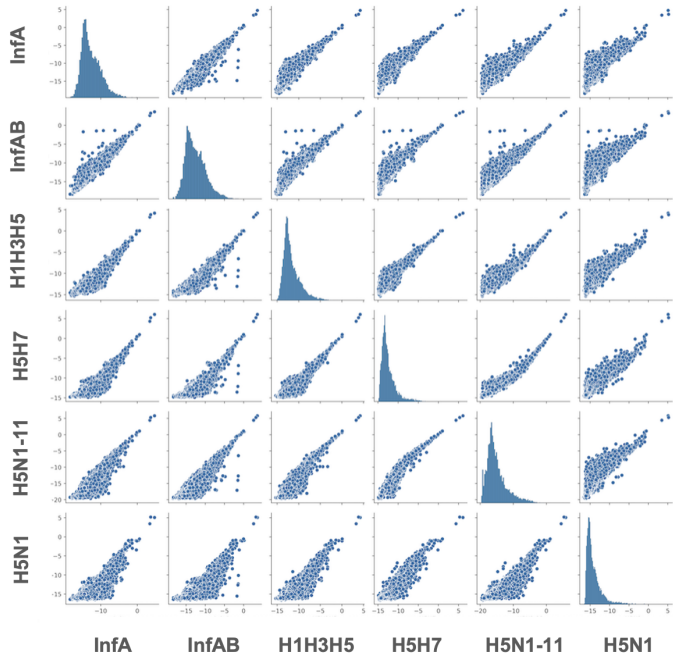

Figure 2. Correlation of EVE scores across different alignments

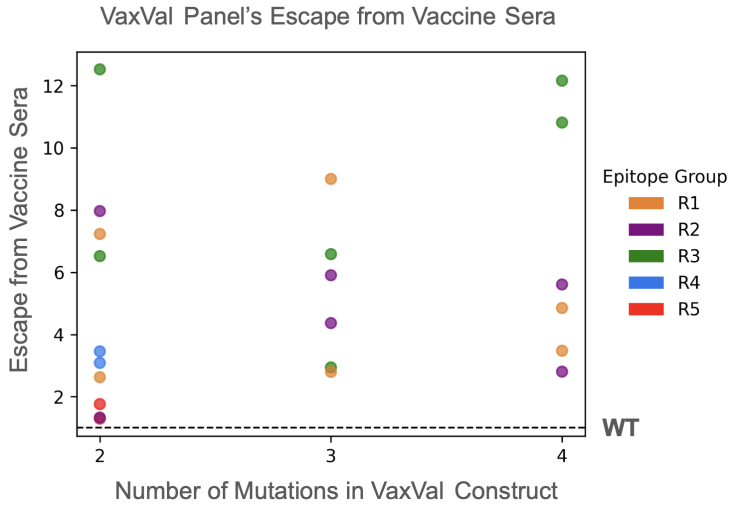

Figure 3. Number of mutations per VaxVal construct does not correlate with relative escape from vaccine sera.

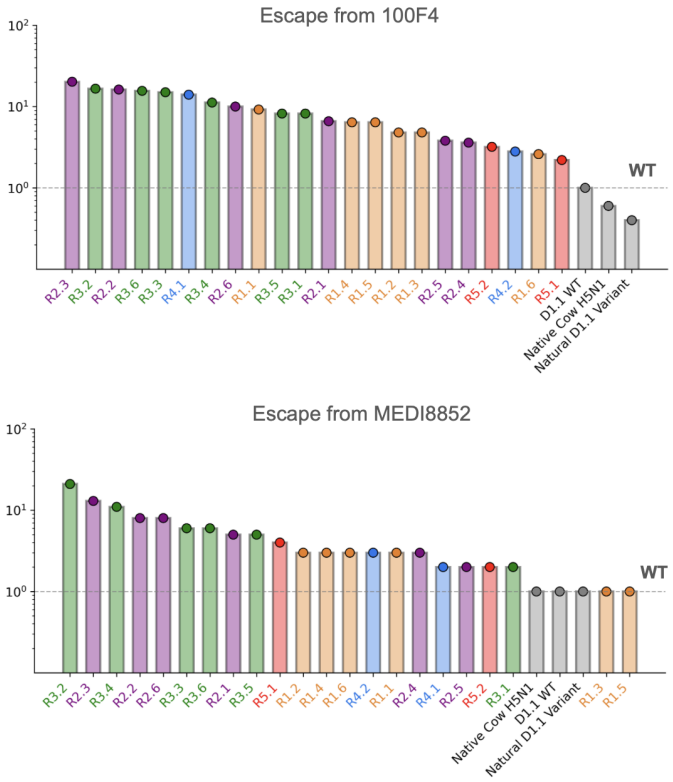

Figure 4. Relative escape of VaxVal panel from 100F4 (head antibody) and MEDI8852 (stem antibody)

C. Supplementary Tables

Variant	Mutations relative to D1.1
D1.1	–
BC Teen V1	A**156**T
Native Cow	I11V, A52T, L120M, L**131**Q, T211I, V**226**A, S336N, R341K, D491N, I526V

Table 1. Three circulating variants were tested for neutralization and infectivity, along with the VaxVal panel. Bolded positions were also mutated in constructs in the VaxVal panel.