or $pT_H17$ populations into vancomycin-treated mice and evaluated their capacity to reestablish the CTX-mediated tumor growth retardation. Ex vivo propagated $pT_H17$ exhibited a pattern of gene expression similar to that expressed by CTX-induced splenic $CD4^+$ T cells in vivo (fig. S11). Only $pT_H17$, but not $T_H17$ cells, could rescue the negative impact of vancomycin on the CTX-mediated therapeutic effect (Fig. 4G). These results emphasize the importance of $pT_H17$ cells for CTX-mediated anticancer immune responses.

Although much of the detailed molecular mechanisms governing the complex interplay between epithelial cells, gut microbiota, and intestinal immunity remain to be deciphered, the present study unveils the unsuspected impact of the intestinal flora on chemotherapy-elicited anticancer immune responses. Our data underscore new risks associated with antibiotic medication during cancer treatments, as well as the potential therapeutic utility of manipulating the gut microbiota.

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### Supplementary Materials

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# Substitutions Near the Receptor Binding Site Determine Major Antigenic Change During Influenza Virus Evolution


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**The molecular basis of antigenic drift was determined for the hemagglutinin (HA) of human influenza A/H3N2 virus. From 1968 to 2003, antigenic change was caused mainly by single amino acid substitutions, which occurred at only seven positions in HA immediately adjacent to the receptor binding site. Most of these substitutions were involved in antigenic change more than once. Equivalent positions were responsible for the recent antigenic changes of influenza B and A/H1N1 viruses. Substitution of a single amino acid at one of these positions substantially changed the virus-specific antibody response in infected ferrets. These findings have potentially far-reaching consequences for understanding the evolutionary mechanisms that govern influenza viruses.**


Influenza A/H3N2 virus is a major cause of morbidity and mortality in humans and poses a considerable economic burden (*1*, *2*). Vaccination is the primary method to reduce this public health impact. The hemagglutinin (HA) surface glycoprotein is the main component of influenza vaccines, and antibodies to HA can prevent serious illnesses (*3*). However, influenza viruses can escape from antibody-mediated neutralization by accumulating mutations in HA in a process called antigenic drift, and as a consequence influenza vaccines require frequent updates. Several recent studies have focused on the identification of conserved domains of HA as targets of virus-neutralizing antibodies to circumvent this problem (*4*–*7*). Other recent work has focused on identifying the mechanisms of antigenic drift (*8*, *9*) and on sequence-based prediction to identify positively selected codons (*10*–*13*). This research has been restricted by our limited fundamental insight into the molecular basis of antigenic evolution.

Seminal work in the 1980s identified 131 amino acid positions in five antigenic sites (A to E) on the globular head of HA as main targets for specific antibodies and suggested that antigenic drift is caused by accumulation of amino acid substitutions in these sites (*14*, *15*). This work led to the widely used heuristic that it takes at least four amino acid substitutions, spread between two or more different antigenic sites, to cause substantial antigenic change. Smith *et al.* (*16*) showed that 11 antigenic clusters of viruses emerged during the 35-year period that followed the introduction of the A/H3N2 virus in humans in 1968, each of which was subsequently replaced by viruses with distinct antigenic properties. Between 1 and 13 amino acid substitutions were associated with each of the antigenic cluster transitions. Almost all of these cluster-difference substitutions were in the antigenic sites (*16*). Here, we investigated which of these substitutions actually caused the antigenic change.

We selected a representative virus from each antigenic cluster. The HA1 subunit amino acid sequence, which comprises the globular head domain of HA including the receptor binding site (RBS), of each representative virus was identical to the consensus sequence for all strains from the respective cluster (*17*). The consensus HA genes, representing natural circulating viruses, were used to make recombinant viruses in the context of the A/Puerto Rico/8/1934 reference virus (*18*). We also produced chimeric viruses with the full HA1 or with HA1 positions 109 to 301 of each antigenic cluster consensus strain in the context of HA of the Sichuan 1987 cluster consensus virus (fig. S1). The antigenic properties of all viruses were analyzed in hemagglutination inhibition (HI) assays using a panel of 8 to 16 ferret antisera raised against A/H3N2 viruses between 1968 and 2006 (table S1). The wild-type, recombinant, and chi-


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meric viruses all had similar antigenic properties in the HI assay (figs. S2 and S3). Thus, HA1 positions 109 to 301 determined the antigenic phenotype of representative A/H3N2 strains circulating between 1968 and 2003.

Each of the 54 cluster-difference substitutions within positions 109 to 301 of the HA1 region (table S2) was introduced independently into the corresponding consensus HA and tested for antigenic effect. If no single substitution induced the antigenic change to the subsequent cluster, combinations of substitutions were tested. We call the subset of cluster-difference substitutions that were responsible for the major antigenic change between clusters "cluster-transition" substitutions. The cluster-transition substitutions were also tested in reverse. For example, each of the 11 cluster-difference substitutions between the EN72 and VI75 clusters were introduced individually in the EN72 consensus virus. We found that substitution 189QK alone caused the change in antigenic phenotype from EN72-like to VI75-like. The reverse substitution 189KQ was then introduced into the VI75 consensus HA and resulted in reversion of the antigenic phenotype to EN72-like (fig. S4B).

Surprisingly, 7 of the 10 cluster transitions were caused by only a single amino acid substitution (Fig. 1 and table S3). Two cluster transitions were caused by two substitutions, and one by three substitutions. In two of the three cluster transitions for which more than one substitution was required for the full antigenic change, one substitution was responsible for the majority of the antigenic change and the additional substitution(s) had a relatively modest effect on the antigenic phenotype (Fig. 1; fig. S4, C and E; and table S2). For the remaining transition, the effect caused by the single substitution could not be determined

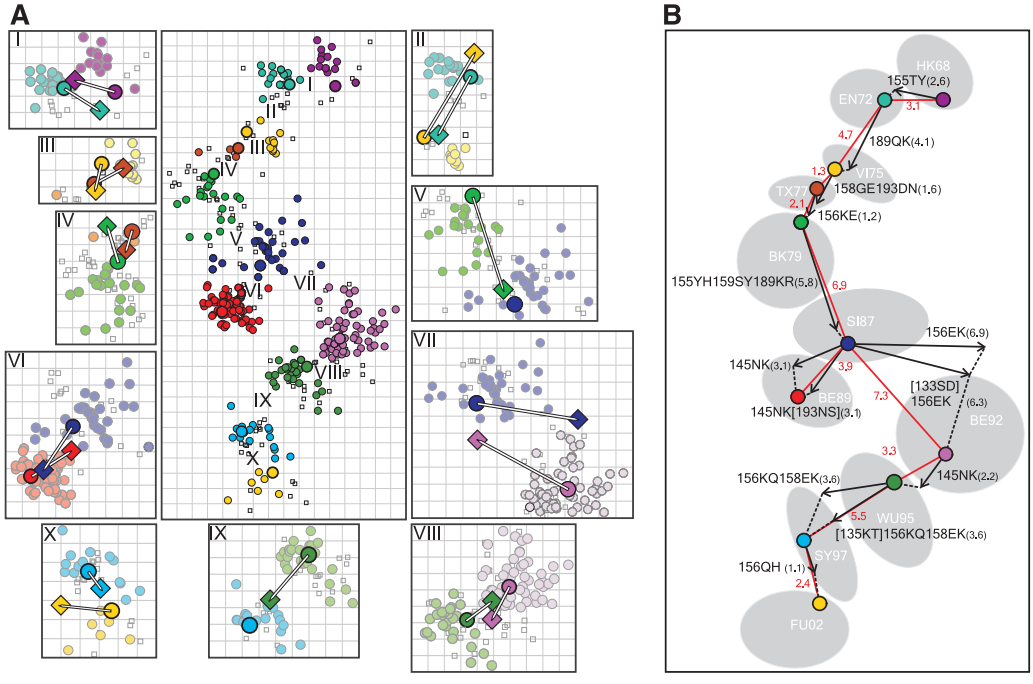


**Fig. 1. Antigenic effect of cluster-transition substitutions.** (**A**) Antigenic maps of A/H3N2 virus evolution and cluster transition mutants. The central map provides an overview in which antisera and epidemic strains are indicated by open squares and colored circles, respectively, and consensus viruses by larger colored circles. Both vertical and horizontal axes represent antigenic distance. The spacing between gridlines is one antigenic unit distance, corresponding to a factor of 2 difference in the HI assay. Cluster names are as indicated in (B). Maps I through X show each cluster transition in more detail. Diamonds indicate the position of viruses with cluster-transition substitutions. (**B**) Overview of cluster-transition substitutions. The clusters are named after the first vaccine strain in the cluster, with letters and digits referring to location and year of isolation (HK, Hong Kong; EN, England; VI, Victoria; TX, Texas; BK, Bangkok; SI, Sichuan; BE, Beijing; WU, Wuhan; SY, Sydney; FU, Fujian). Colored circles indicate the consensus viruses. The gray background shapes indicate the cloud of strains that make up an antigenic cluster. The red line represents the evolutionary path from the HK68 to the FU02 consensus virus. Antigenic distances between consensus viruses are shown in red. Arrows indicate antigenic distance and direction of the cluster-transition amino acid substitutions (abbreviations: D, Asp; E, Glu; G, Gly; H, His; K, Lys; N, Asn; Q, Gln; R, Arg; S, Ser; T, Thr; Y, Tyr). Antigenic distance between the mutant and consensus virus is indicated in parentheses. Substitutions between square brackets are accessory substitutions, which changed direction toward the subsequent cluster (Fig. 2, figs. S6 and S10, and table S3) and did not significantly add to the antigenic distance.

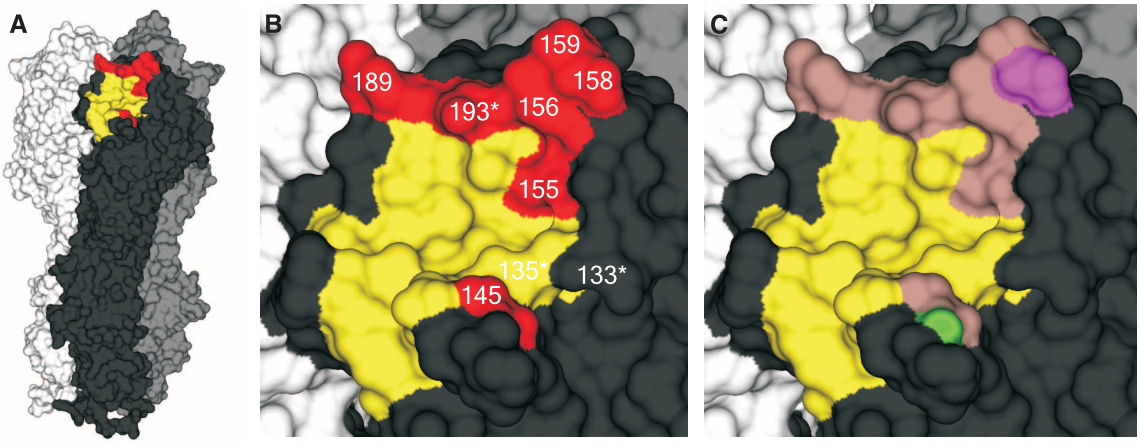


**Fig. 2. Positions of the cluster-transition amino acid substitutions indicated on an A/Aichi/2/1968 HA trimer.** The three monomers are shown in black, white, and gray; the RBS is in yellow. (**A** and **B**) The positions responsible for A/H3N2 cluster transitions are shown in red. An asterisk indicates accessory substitutions (fig. S10). Position 193 is both a cluster-transition substitution and an accessory substitution (Fig. 1B). (**C**) Positions of amino acid substitutions responsible for antigenic change of influenza A/H1N1 and B virus are shown in green and magenta, respectively. The positions responsible for cluster transitions of A/H3N2 virus are shown in light brown.

(fig. S4I). We further tested the results for all cluster transitions using virus neutralization assays and obtained similar results (figs. S5 and S6 and table S2). In summary, we found that at least 9 of 10 A/H3N2 cluster transitions over the 35-year period were predominantly caused by single amino acid substitutions.

Strikingly, all the cluster-transition substitutions occurred at only seven positions, immediately adjacent to the RBS (Fig. 2). Six of seven positions align to form an antigenic ridge on the periphery of the RBS; the other, at position 145, is located on a loop that is partly involved in receptor binding. Five of seven key positions were involved in a cluster transition at least twice (Fig. 1 and table S3).

Substitutions responsible for major antigenic change were located exclusively in antigenic sites A (position 145) and B (positions 155, 156, 158, 159, 189, and 193), with none in sites C, D, or E. Our results do not contradict the studies that found 131 positions that can cause antigenic change and that define sites A to E (*14*, *15*). Rather, we show that nature has, during 35 years of A/H3N2 virus circulation in humans, selected for the substantial antigenic changes caused by substitutions at only seven of these 131 positions; this is an important change in our understanding of the antigenic evolution of seasonal influenza viruses. Even though antibodies have been found to bind many regions of the HA, the location of the key positions for antigenic change on the periphery of the RBS suggests that RBS region-specific antibodies play the critical role in neutralizing influenza A/H3N2 viruses.

To test the surprising finding that the antigenic phenotype was determined by the amino acids at just seven positions, we attempted to change the phenotype of the HK68 consensus virus to become FU02-like by introducing only the five cluster-transition substitutions that differ between the HK68 and FU02 antigenic clusters (table S4). This test compares the effect of substitutions at just five positions (HK68+5) to the 54 substitutions that occurred over 35 years between HK68 and FU02, 47 of which were in antigenic sites. Remarkably, we found that the HK68+5 mutant was FU02-like antigenically; it had an HI titer a factor of 3 lower than that of the FU02 consensus virus to a FU02 antiserum (fig. S7 and table S4). Although HK68+5 had a substantially (factor of 20) reduced titer with the HK68 antiserum (by a factor of 20), it retained some residual low reactivity with the HK68 serum. In a similar experiment, we incrementally introduced cluster-transition substitutions into the HK68 consensus virus. The antigenic properties of the mutants matched those of the consensus viruses from subsequent clusters other than in their HI titers to one of the two HK68 sera. After introduction of all cluster-transition substitutions between the first five clusters, we were unable to rescue the mutant virus. In summary, even for highly divergent strains with as many as 54 substitutions and 35 years of evolution between them, only the amino acid substitutions at key positions determined the majority of the antigenic phenotype.

We next tested the effect of a cluster-transition substitution in HA on the antibody response in an infected host. Two groups of three ferrets each were inoculated with either the wild-type HK68 consensus virus or HK68 containing cluster-transition substitution 155TY, and antisera were collected 2 weeks later. Each antiserum was titrated in the HI assay to 31 viruses belonging to the HK68, EN72, or VI75 antigenic clusters (*16*). Relative to its reactivity with HK68-like viruses, antisera to the HK68 consensus virus had a factor of 25 (4.6 $\log_2$) reduction in reactivity to EN72-like strains and had no reactivity to the majority of the VI75-like viruses. In contrast, antisera to HK68 155TY had only a factor of 4 (2.1 $\log_2$) reduction in titers to EN72-like strains but had substantial titers to the viruses from the VI75 cluster (Fig. 3 and fig. S9). Thus, a single cluster-transition substitution can also substantially increase reactivity of the antiserum raised to the mutant virus to strains from subsequent antigenic clusters while maintaining reactivity to the antigenic cluster of origin.

The identification of key influential positions in HA and single amino acid substitutions responsible for major antigenic changes provides a plausible explanation for the previously described punctuated nature of the antigenic evolution of influenza A/H3N2 virus (*16*): 9 out of 10 cluster transitions were predominantly caused by a single amino acid substitution.

Although we found that almost 90% of HA substitutions associated with cluster transitions had little or no measurable antigenic effect, these findings do not imply that substitutions away from the RBS are phenotypically neutral. Some may be neutral hitchhikers carried along by chance, but others may collectively add to the decreased recognition by antibodies raised to a strain in an earlier cluster (Fig. 3 and figs. S7 and S8), or they may be compensatory mutations necessary to retain function.

Given the high mutation rate of influenza virus and the observation that single amino acid substitutions are sufficient to cause antigenic cluster transitions, it is surprising that new antigenic clusters appear as slowly as they do—on average every 3.3 years (*16*). One hypothesis is that antigenic change has an intrinsic fitness cost that slows down the antigenic evolution of the virus. We found that several mutant viruses with substitutions near the RBS could not be rescued (fig. S4, E and I, and fig. S8). These results suggest that introduction of mutations on the periphery of the RBS can affect HA function and that co-mutations may be crucial to retain viral fitness. The requirement for co-mutations that offset the fitness cost of antigenic evolution could provide an explanation for the paradox of high mutation rate and slow antigenic evolution.

Almost all substitutions responsible for cluster transitions resulted in substantial changes in

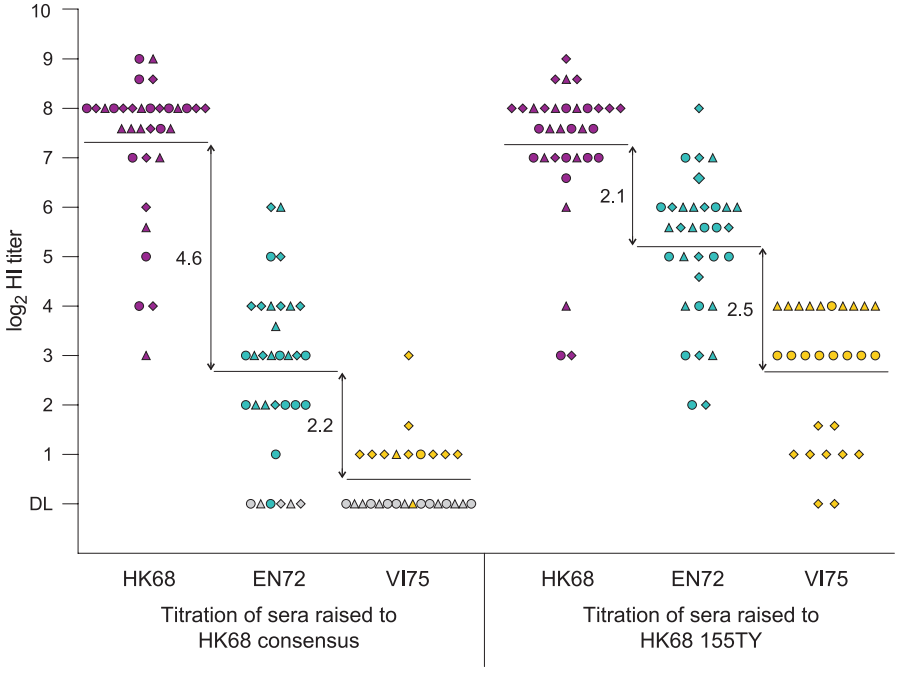


**Fig. 3. Effect of a cluster-transition substitution on the antibody response of ferrets.** Groups of three ferrets were inoculated with HK68 consensus virus or HK68+155TY, and antisera were tested to strains from the HK68 (purple), EN72 (cyan), or VI75 (yellow) clusters of Fig. 1. Circles, triangles, and diamonds mark the three individual antisera. Black horizontal lines show the mean $\log_2$ HI titer. DL indicates the detection limit of the HI assay at the starting dilution (1/20) we used. Reactivity below detection was set to the value of the detection limit to calculate the means; these points are indicated in gray. The $\log_2$ reduction in mean reactivity to the different strains is indicated between the groups.

the biophysical properties of the amino acids involved. For instance, in nine cluster transitions at least one charge change was involved, and substantial volume changes were involved in three transitions (table S5). The amino acid composition of the key positions seems limited by their exposed nature. Hydrophobic amino acids are typically located on the interior of a protein, where they can be shielded from solvent access. Tyrosine, which is only partially hydrophobic because of the hydroxyl group on its aromatic ring, was the only hydrophobic amino acid observed on the key positions. The necessity for maintaining a functional HA structure as well as escape from neutralizing antibodies may have restricted the range of possible amino acids.

The number of potential glycosylation sites on the A/H3N2 virus HA has steadily increased since 1968 (*19*), and the presence of carbohydrate side chains has been associated with shielding of antibody epitopes (*20*, *21*). A total of 14 HA positions are associated with glycosylation of the A/H3N2 viruses that circulated between 1968 and 2003. Two positions, 133 and 144, are located adjacent to the RBS. However, changes in glycosylation did not coincide with cluster transitions, and viruses belonging to the same antigenic cluster often had different glycosylation states (*22*). In agreement with these observations, the cluster-transition substitutions neither introduced nor deleted any glycosylation sites, and glycosylation was therefore not directly involved in the antigenic changes between the clusters. The location of the majority of the potential glycosylation sites away from the RBS could be the reason why changes in glycosylation have not played a major role in the antigenic change of A/H3N2 viruses.

To further expand these results, we examined the most recent antigenic cluster transitions in the other human seasonal influenza viruses: the B/Yamagata and B/Victoria lineages of the B viruses, and the pre-2009 A/H1N1 viruses (the current A/H1N1pdm09 viruses are yet to undergo a cluster transition). We found the same results as for the A/H3N2 viruses: The major antigenic change was caused by a single amino acid substitution in the corresponding region close to the RBS (Fig. 2C and figs. S11 to S13).

We find that although human A/H3N2 seasonal influenza viruses have fixed amino acid substitutions at 54 positions in antigenic sites, substitutions at only seven of these locations have been responsible for the major antigenic changes in these viruses to date. Moreover, these locations are all near the RBS of the HA, which suggests the mechanism for slowing the antigenic evolution of these viruses could be a reduction in receptor binding function. This small number of critical positions, and the restricted amino acid usage involved in antigenic cluster transitions, suggests that possibilities for important antigenic change of seasonal influenza viruses may be more restricted than previously thought, with potentially far-reaching consequences for understanding the underlying evolutionary mechanisms governing such viruses.

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### Supplementary Materials

www.sciencemag.org/content/342/6161/976/suppl/DC1
Materials and Methods
Figs. S1 to S13
Tables S1 to S6
References (*23*–*29*)


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# Yeast Reveal a "Druggable" Rsp5/Nedd4 Network that Ameliorates α-Synuclein Toxicity in Neurons


**Daniel F. Tardiff,[1] Nathan T. Jui,[2] Vikram Khurana,[1,3] Mitali A. Tambe,[4] Michelle L. Thompson,[5]* Chee Yeun Chung,[1] Hari B. Kamadurai,[6] Hyoung Tae Kim,[7] Alex K. Lancaster,[1]† Kim A. Caldwell,[5] Guy A. Caldwell,[5] Jean-Christophe Rochet,[4] Stephen L. Buchwald,[2] Susan Lindquist[1,8]‡**



α-Synuclein (α-syn) is a small lipid-binding protein implicated in several neurodegenerative diseases, including Parkinson's disease, whose pathobiology is conserved from yeast to man. There are no therapies targeting these underlying cellular pathologies, or indeed those of any major neurodegenerative disease. Using unbiased phenotypic screens as an alternative to target-based approaches, we discovered an *N*-aryl benzimidazole (NAB) that strongly and selectively protected diverse cell types from α-syn toxicity. Three chemical genetic screens in wild-type yeast cells established that NAB promoted endosomal transport events dependent on the E3 ubiquitin ligase Rsp5/Nedd4. These same steps were perturbed by α-syn itself. Thus, NAB identifies a druggable node in the biology of α-syn that can correct multiple aspects of its underlying pathology, including dysfunctional endosomal and endoplasmic reticulum–to-Golgi vesicle trafficking.


Phenotypic cell–based drug screens are a powerful yet underused approach to identify lead compounds and probe the underlying cellular pathologies that cause human disease (*1*). Such unbiased screens may be particularly helpful for neurodegenerative diseases (NDs), such as Parkinson's disease (PD) and Alzheimer's disease (AD), for which the molecular underpinnings of disease remain unclear. However, establishing robust neuronal phenotypes amenable to high-throughput screening and subsequent target identification remains a challenge. To bridge this gap, we exploited yeast cells that express ND-causing proteins to recapitulate salient cellular pathologies. α-Synuclein (α-syn), for example, causes derangements in vesicle trafficking, metal ion homeostasis, and mitochondrial function that are associated with α–synucleinopathies, such as PD (*2*). The resulting growth inhibition greatly facilitates robust high-throughput screening.

We recently screened ~190,000 compounds for their ability to restore the growth of cells




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**Substitutions Near the Receptor Binding Site Determine Major Antigenic Change During Influenza Virus Evolution**

Björn F. Koel, David F. Burke, Theo M. Bestebroer, Stefan van der Vliet, Gerben C. M. Zondag, Gaby Vervaet, Eugene Skepner, Nicola S. Lewis, Monique I. J. Spronken, Colin A. Russell, Mikhail Y. Eropkin, Aeron C. Hurt, Ian G. Barr, Jan C. de Jong, Guus F. Rimmelzwaan, Albert D. M. E. Osterhaus, Ron A. M. Fouchier and Derek J. Smith (November 21, 2013)

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Editor's Summary

**Flu Drift Limited**

Five antigenic sites in the virus surface hemagglutinin protein, which together comprise 131 amino acid positions, are thought to determine the full scope of antigenic drift of influenza A virus. **Koel *et al.*** (p. 976) show that major antigenic change can be caused by single amino acid substitutions. These single substitutions substantially skew the way the immune system "sees" the virus. All substitutions of importance are located next to the receptor-binding site in the hemagglutinin. Because there are few positions of importance for antigenic drift, there are strict biophysical limitations to the substitutions at these positions, which restricts the number of new antigenic drift variants at any point in time. Thus, the evolution of influenza virus may be more predictable than previously thought.



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