viruses

MDPI

Review

Targeting Hemagglutinin: Approaches for Broad Protection against the Influenza A Virus

Yun Zhang [1,†], Cong Xu [2,†], Hao Zhang [1], George Dacai Liu [3], Chunyi Xue [1] and Yongchang Cao [1,*]

[1] State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou 510006, PR China; zhangyun6@mail.sysu.edu.cn (Y.Z.); zhanghao5@mail.sysu.edu.cn (H.Z.); xuechy@mail.sysu.edu.cn (C.X.)

[2] Research Center of Agricultural of Dongguan City, Dongguan 523086, China; wswdg@126.com

[3] Firstline Biopharmaceuticals Corporation, 12,050 167th PL NE, Redmond, WA 98052, USA; georgeliu2100@yahoo.com

* Correspondence: caoych@mail.sysu.edu.cn; Tel.: +86–020-39332938

† These authors contributed equally to this work.

Received: 10 April 2019; Accepted: 27 April 2019; Published: 30 April 2019

Abstract: Influenza A viruses are dynamically epidemic and genetically diverse. Due to the antigenic drift and shift of the virus, seasonal vaccines are required to be reformulated annually to match with current circulating strains. However, the mismatch between vaccinal strains and circulating strains occurs frequently, resulting in the low efficacy of seasonal vaccines. Therefore, several "universal" vaccine candidates based on the structure and function of the hemagglutinin (HA) protein have been developed to meet the requirement of a broad protection against homo-/heterosubtypic challenges. Here, we review recent novel constructs and discuss several important findings regarding the broad protective efficacy of HA-based universal vaccines.

Keywords: influenza A virus; hemagglutinin; universal influenza vaccine

1. Introduction

According to the World Health Organization (WHO), influenza A viruses (IAVs) annually cause about 3 to 5 million cases of severe illness and approximately 290,000 to 650,000 respiratory deaths worldwide [1]. IAV is a member of the *Orthomyxoviridae* family. The viral genome is a negative-sense, single-stranded RNA possessing eight segments, which encodes at least 10 proteins including polymerase basic 1 (PB1), PB2, polymerase acid (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix 1 (M1), M2, nonstructural 1 (NS1) and NS2. The HA and NA proteins present the major surface glycoproteins of the virion, while the NP, PB1, PB2 and PA proteins (P-complex) associated with viral RNA from the viral ribonucleoprotein complex (vRNP) [2]. The RNA polymerase of the virus has no proof-reading activity, thus contributing to rapid small changes of the viral genome, resulting in a high mutation rate of IAVs. The phenomenon of small changes in the viral genome is referred to as "antigenic drift" [3]. The accumulated mutations in the IAV genome lead to the high plasticity of the HA protein. Based on the genetical differences of the HA amino acid sequences, IAVs are phylogenetically classified into two groups: group I and group II [4,5]. Based on the genetic and antigenic variability of the HA and NA proteins, the viruses were further divided into 18 distinct HA subtypes and 11 NA subtypes [6]. Among different HA subtypes, H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18 belong to group I, whereas H3, H4, H7, H10, H14, H15 belong to group II. Phylogenetically, group I is classified into three clades and group II is divided into two clades [7,8]. Genetically, the similarity of HA amino acid sequences within one subtype was estimated to be more than 90% [9], and about 60%–74% between the subtypes within one group,

while the similarity between different groups was only 40% to 44% [10,11]. The H17 and H18 subtypes were recently isolated from bats [12].

In general, IAVs are species specific. The natural reservoir of the viruses is wild birds and waterfowl. Therefore, almost all the HA and NA recombination could be identified in avian species. H1, H2, H3, H5, H6, H7, H9 and H10 subtypes have been found in humans, while H1N1 and H3N2 subtypes are currently epidemic. The H1 and H3 subtypes combined with either N1 or N2 subtypes have been detected in swine, and the H3 subtype is epidemic in horses and dogs. Among avian influenza viruses (AIVs), the H5N1, H5N6 and H7N3 subtypes are highly pathogenic, while H9N2, H7N9, H6N1, H10N8, H7N2, and H7N3 are low-pathogenic [13]. In addition, the insertion of a polybasic cleavage motif in the H2, H4, H6, H8, H9, and H14 subtypes could lead to a highly pathogenic phenotype [14–16]. Furthermore, among the different subtypes of AIVs, H5N1 and H7N9 subtypes have posed great threats to public health. Importantly, the increasing numbers of H7N9 human infections suggest the virus remains a potential pandemic threat [17]. So far, of all AIV infections, very limited cases of human-human transmission were reported [18]. However, taking the rapid mutation and recombination rate of the viral genome into consideration, AIVs still possess the risk of pandemic potential, thus posing great challenges to public health [19–21].

The mixed infection of different IAV subtypes leads to the generation of re-assorted viruses. Several researchers have explored the reassortment of two different influenza subtypes in cells or animals [22–24]. This phenomenon is referred to as "antigenic shift" [25]. Because of the absence of pre-existing immunity in the human immune system, the re-assorted IAVs (usually from avian and porcine origins) contribute to irregular pandemics [26,27], and caused at least the last three pandemics [28]. These pandemic strains are antigenically distinct from the circulating seasonal strains.

Vaccination is an efficient and cost-effective way to prevent and control the influenza virus infection in both human and animal populations [29]. Current influenza vaccines are effective when the antigenicity of the vaccine strain is closely matched with the circulating strain. As a result of antigenic drift, traditional vaccines need to be reformulated annually in order to elicit protective antibody responses against the current circulating strain. Recently, data mining techniques were applied to distinguish pandemic from seasonal strains [30], which could provide clues to vaccine strain selection. However, since a long period is required from epidemic strain identification to vaccine production and distribution (usually more than six months), prevention by traditional vaccines at an early stage of the pandemic would be impossible. Therefore, to provide long-lasting and broad protection against multiple IAV strains, the global scientific community is attempting to develop universal influenza virus vaccines. In this review, we will discuss HA-based approaches, including our own, on designing universal influenza vaccines in recent years.

2. Hemagglutinin

2.1. The Structure of the Hemagglutinin

Hemagglutinin (HA) is a type I glycoprotein, which is the most abundant transmembrane protein on the surface of influenza viral particles. Each IAV virion contains approximately 500 molecules of HA [31]. Among all IAV viral proteins, HA evolves at the highest rate [32]. However, although the sequences of HAs from different IAV subtypes share a low sequence identity, they present similar protein structure [33].

Since the first crystal structure of HA was solved, more than 350 entries are available now in the Protein Data Bank (PDB), and new structures of HA have continued to be solved in the past years. Recently, the structure of H15 HA was solved in complex with an avian receptor analog 3'SLN (NeuAcα2–3Galα1–4GlcNAc) [34].The matured functional HA protein is a homotrimer composed of a globular head (most HA1) bearing the Receptor Binding Site (RBS) and antigenic determinants and a stalk region (most HA2 with some residues from the N- and C- termini of HA1) [35]. The HA1 is highly variable except the RBS [36,37]. The RBS contains four highly conserved amino acid residues (Y98, W153, H183 and Y195) surrounded by four structural elements (130-loop, 150-loop, 190-helix

and the 220-loop) [34,38]. It is responsible for viral infectivity and serves as a major determinant of host infection [39]. Single amino acid substitutions at positions adjacent to the RBS resulted in an antigenic drift [40]. The HA2 is relatively conserved [33]. One of the most conservative regions in the stalk region is a 55 amino acid long alpha helix (LAH) [41,42]. A 3D-structure of the HA-trimer is illustrated in Figure 1.

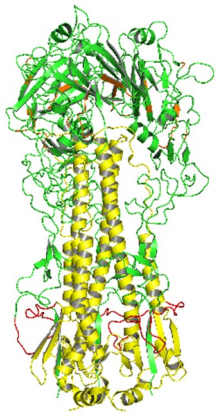

Figure 1. A 3D-structural diagram of the hemagglutinin (HA) trimer. The representative virus was A/X-31(H3N2), and the sequence was obtained from GenBank (Accession No. P03438). The structure of HA was constructed by Swiss-Model (https://www.swissmodel.expasy.org/). The HA head is marked in green. The HA stalk is marked in yellow. The fusion peptide is marked in red, and the Receptor Binding Site (RBS) is marked in orange.

Each HA monomer is comprised of three structural domains: one hydrophilic ectodomain, one small transmembrane domain, and one cytoplasmic domain. In recent years, the effect of HA transmembrane (TM) domain in HA structure and function has drawn attention. Since the TM domain mainly contains hydrophobic residues, it is highly conserved and responsible for the insertion of the HA into a viral envelope. Furthermore, it is important for the stability and structure of the HA trimer [43].

The high plasticity of HA is responsible for the viral escape from immune neutralization and antiviral drugs. To evade the host immune system, HA undergoes continuous structural changes by introducing mutations, take H3N2 for instance [44]. Over the past years, the H3N2 HA has accumulated at least 75 substitutions, consisting of 13% of the entire protein. Most mutations are located within or immediately proximate to the RBS [40,45]. These changes are responsible for determining antigenic phenotype and HA binding. Further research on residues 158–160 suggests that the residue 158 is important for the H3N2 HA backbone structure and the structural changes of this region coincides with H3N2 HA1 evolution [46]. Similar observations are found in H1N1 as well as influenza B viruses [40,47,48]. In addition, the number of N-glycosylation sites in the H3N2 HA head domain had been increasing in the past years [49,50]. The added N-glycosylation sites could affect receptor binding when they are proximal to the RBS [51–54]. Understanding the structural properties of the HA may further help in elucidating the correlation of viral evolution and infection, thus contributing to antiviral therapeutic strategies.

2.2. Binding Affinity of the Hemagglutinin

Mammalian cells are covered by different types of glycans, some of which are connected to sialic acid (SA). In humans, the main SA is N-acetylneuraminic acid. The HA is responsible for host cell attachment through binding to the SA, resulting in cellular fusion and host cell entry. The overall affinity of the binding is related to the HA and NA subtypes of the virus as well as the form and density of the SA on the membrane. To understand the molecular mechanism underlying, different platforms based on streptavidin-modified surfaces for the detection of viruses were applied in order to explore the interaction between viruses and cell receptors [55,56]. To control the SA density at the

membrane, self-assembled monolayers (SAMs) or (fluid) supported lipid bilayers (SLBs) were applied to obtain static or mobile layers, respectively [57–59]. Recently, a SA receptor-presenting the SLB platform was reported to be utilized to analyze the multivalent interaction between the HA and host cells, as well as other cellular responses [60].

Critical amino acids located in the RBS determine the HA affinity for each receptor. For instance, human IAV strains contain leucine at position 226, which preferentially binds to SA α-2,6 galactose, while avian IAV strains contain glutamine at the same position, resulting in a preference in binding to SA α-2,3 galactose [61,62]. The α-2,3 and α-2,6 SA are present in both the respiratory and intestinal tract of avian species [63]. In addition, the α-2,3 SA receptors are present on epithelial cells in birds and in the lower respiratory tract (LRT) in humans (type II pneumocytes and non-ciliated cells), which might be the reason why HPAI viruses could result in a severe infection in humans [64]. Furthermore, sialidase treated cells were also shown to be sensitive to IAVs, suggesting other receptors may also contribute to RBS recognition [65,66]. Considering the evidence that Neu5Acα-2–8Neu5Acα2–8Neu5Ac, Galβ- and GlcNAcβ-terminated glycans could be recognized by IAVs, the polysaccharides other than *N*-acetylneuraminic acid might be more important in IAV infections [67].

2.3. Fusion Function of the Hemagglutinin

The fusion process is related to the maturation of the HA protein by the proteolytic cleavage of HA0 precursor into two disulfide-linked HA1 and HA2 subunits. Trypsin-like serine endoproteases are responsible for the cleavage of the precursor HA0 [68,69]. High pathogenic H5 and H7 subtypes contain a polybasic cleavage site in the HA, which could also be catalyzed by protease furin [70,71]. The HA1 is responsible for the viral attachment to the host cell by recognizing sialylated glycans. After receptor-mediated endocytosis, the fusion peptide, located at the N-terminus of the HA2, is subsequently inserted into the lipid bilayer of the endosomal membrane, thus allowing the fusion of the viral and endosomal membranes, resulting in the release of the viral content into the cytoplasm [5,72,73].

The fusion peptide containing 23 amino acids (HAfp23) with a fusogenic activity adopts a helical-hairpin structure [74]. Recently, by means of molecular dynamics simulations and spectroscopic measurements, three C-terminal W21-Y22-G23 within the HAfp23 were shown to promote the hairpin structure, which is postured in a perpendicular orientation to the membrane plane [75]. Together with HAfp, the HA TM domain may also be involved in the post-fusion structure, considering the fact that the two domains are inserted in the membrane after viral fusion [76].

Other factors such as pH and the physical properties of the membrane also contribute to the fusion process. For instance, pH stability plays an important role in the membrane fusion function of HA [77] as well as in the pathogenicity and airborne transmissibility of the virus [78]. The acidic pH environment inside the endosomes triggers the "loop-to-helix" transition of HA2, resulting in merging of the viral and endosomal membranes [72,79]. Since cholesterol plays an important part in the rigidity of the membranes, the addition of cholesterol has been shown to increase fusion activity [80]. By studying influenza virus-like particles (VLPs) containing HA hemifusion mutant and liposome mixtures, researchers proposed two pathways of HA-induced membrane fusion, and cholesterol concentration plays a role as a switcher between pathways through its negative spontaneous curvature [81].

2.4. Stability and Immunity of the Hemagglutinin

The stability of the HA trimer was shown to be responsible for membrane fusion in acidic environments [82,83]. Several reviews have summarized the relationship between HA mutants and its stability [82,83]. In general, HA stability is determined by specific amino acids throughout the HA1 and HA2 subunit sequences. For instance, a point mutation in H5N1 HA could alter the acid stability of the protein, the mutated HA also presented enhanced high-temperature resistance [84], suggesting a more stable HA could contribute to a prolonged virus half-life. Amino acids at positions 205 and

402 in the HA1 and HA2 subunits of the H1N1 HA, respectively, are also important for the stability of the protein [85].

Researchers also suggest the function of HA transmembrane domain in HA stability. When glycosyl-phosphatidyl inositol (GPI) was utilized for the HA transmembrane domain, structural changes were found compared to the wild type HA protein and the fusion ability was impaired [86]. Only HA monomers were detected in GPI-transmembrane-replaced HAs [87]. Sequence analysis further confirmed conserved cysteine residues (Cys540 and Cys544) in all H3 strains. Researchers suggest that disulfide links formed between these cysteine residues are critical for HA trimerization [88,89]. In addition, the mutation of Cys555, Cys562, and Cys565 at the cytoplasmic tail (CT) of H3 HA did not affect trimer formation as shown in cryo-ET [90]. Researchers suggest that disulfide bonds between these cysteine residues (Cys540 and Cys544) of the transmembrane may contribute to the structural stability and functional activity of the HA trimers [91]. Future work is required to elucidate the formation of these disulfide bonds. Furthermore, stearate is attached to one cysteine in the transmembrane domain, and the cysteine is further *S*-acylated [92]. Palmitoylation, including phosphorylation and acetylation, plays a critic role in protein stability. Researchers suggested that HA acylation could modulate membrane curvature and affect HA-mediated membrane fusion [90]. When the TM domain was absent, HA trimerization was initiated by the interactions between the HA1 N-terminal Ile–Cys–Ile amino acid triads [93].

Owing to the HA epitopes for neutralizing antibody production, the HA protein serves as the primary neutralizing target of the humoral immunity [94,95], thus it is the most important antigen in AIVs. Different IAV strains present different antigenic determinants. A correlation between HA stability and immunity has been discussed for a long time, especially in vaccine design. For instance, a HA-stabilized mutant of H1N1 with lowered activation pH from 5.4 to 5.0 could enhance vaccine stability and infectivity [96]. Moreover, different proteins fused with HA could enhance the stability and result in a broad cross-immunity, take bacteriophage T4 fibritin foldon [97], GCN4PII trimerization [98], and ferritin [99] for instance. A recent study on H5 HA conformational flexibility and antibody binding suggests that HA conformational stability is associated with neutralization sensitivity of the stalk-specific antibodies, while non-epitope residues play a critical role [100]. Taken together, a more stable HA protein may contribute to immunogenicity as well as vaccine production.

3. Broad Spectrum Protection Strategies Targeting the Hemagglutinin

3.1. Antibodies against the Hemagglutinin

Antibodies mediated antiviral effects against viral infections through several mechanisms. Broadly neutralizing antibodies provide a possibility of generating universal influenza immunity in humans [101–103]. To obtain large numbers of antibodies to the virus, comprehensive influenza antibody libraries were created [104]. Two reviews on HA antibodies are recommended here. The broadly neutralizing antibodies against IAVs were comprehensively reviewed by Corti et al [105] and the structural design of small proteins and peptides against the HA was comprehensively reviewed by Wu and Wilson [106]. Antibodies against neuraminidase display broad binding activity and cross-protection as well [107].

Generally, the structure of two antigenic supersites on HA targeted by broadly neutralizing antibodies have been defined. One is the RBS in the head domain [50,108–111], and the amino-acid identity at residue 190 in the HA RBS is suggested to be critical for affinity of the antibodies [112]. The other is at the hydrophobic groove in the stalk domain [113–115]. Antibody engineered to recognize multiple highly conserved epitopes on both head and stalk domains was reported with enhanced virus cross-reactivity and potency [116]. For vectored immunoprophylaxis (VIP), adeno-associated viruses (AAV) were utilized to deliver two characterized broadly neutralizing monoclonal antibodies (F10 and CR6261) through intramuscular injection in mice [117].

3.1.1. Antibodies against the Hemagglutinin Head

After infection, mice were able to produce cross-reactive anti-head antibodies [118]. For the purpose of rapid detection to influenza antibodies, an in vivo human-plasmablast enrichment technique was developed [119]. Considering the high plasticity of the head domain, studies into the conservation domain of HA started from the monoclonal antibodies (mAbs) presenting cross-reaction against HA and a lot of conservative epitopes were identified in this way. For instance, anti-head antibodies CH65, CH67, C05 and F045–092 with a broad neutralizing capacity could serve as "universal" vaccine candidates [50,108–110,120].

Antibodies against the HA head domain could neutralize the virus by either inhibiting cellular receptor binding, membrane fusion, or egress of the virus from infected cells. For instance, antibody H3v-47 was found to inhibit viral egress, thus exhibiting a potent cross-reactive neutralization activity against various H3N2 viruses [121]. Recently, eight mAbs against the H4 HA head were characterized and reported to be cross-protective in the mouse model. These mAbs are non-neutralizing antibodies and active in an antibody dependent cell-mediated cytotoxicity (ADCC) manner [122]. Furthermore, in studies of influenza B virus (IBV), antibodies targeting the highly conserved regions like the RBS could inhibit viral-host recognition and result in the strong cross-protection in mice and ferrets [123–125]. These data could provide guides for the broad protection of IBV vaccines.

Other than the RBS of HA, different vulnerable sites of the head domain are identified. For instance, through investigation on epitopes of antibodies 65C6, 100F4 and AVFluIgG03, four vulnerable sites (VS1–VS4) at the head domain were identified [126,127]. Further study on the VS1 site identified eight pivotal residues, which are critical for antibody binding and neutralization [128]. A study on the 158–170 residues of HA head domain showed that these residues may serve as an epitope responsible for the cross-clade reactivity of the mAbs [129]. These findings may provide a novel perspective for the immunogen design.

3.1.2. Antibodies against the Hemagglutinin Stalk

The HA2 subunit makes up the major part of the HA stalk region and is highly conserved within subtypes [130,131]. Researchers also found that influenza virus infection could elicit antibodies with a broadly neutralizing activity against the stalk region [132,113]. The antibody targeting the stalk region was presented to be able to recognize HAs from both group I and group II IAVs [133]. Moreover, when the passive transfer of sera after H5N1 vaccination was conducted in mice, high levels of anti-stalk antibodies were correlated with protection against H1N1 challenge [134].

Different anti-stalk antibodies have been tested to conduct broad spectrum protection. Vaccines eliciting an antibody response to the stalk region could provide heterosubtypic protection against different HA groups [103,135]. Numerous stalk-based antibodies and immunogens were found to confer heterologous protection in mice [102,119,136–141]. Researchers also developed small protein mimics analogous to the discovered mAbs which bind to the stalk region [142,143]. Anti-stalk antibodies could inhibit either virus entry or viral release by mediating FcγR-dependent effector processes such as antibody-dependent cell-mediated cytotoxicity (ADCC) or complement cytotoxicity [144]. Therefore, these mAbs would provide useful tools for antibody-guided vaccine design as well as therapeutics. A summary of the anti-HA antibodies mentioned in this review is presented in Table 1.

Table 1. The summary of anti-hemagglutinin (HA) Abs.

Antibody	Target	Function	Animal/Cell	Reference
S139/1	HA head	Neutralization of H1, H2, H3, H6, H13, and H16 strains; Reduction of virus titers of H3N2 and H1N1 strains after passive immunization	MDCK cells; Mouse	[118,109]
CH65	HA head	Neutralization of H1N1 strains covering 21 years of antigenic drift	MDCK cells	[110,120]

C05	HA head	Neutralization of H1, H2, H3, and H9 strains; Protection against H1N1 and H3N2 strains after intraperitoneal injection	MDCK cells; Mouse	[108]
CH67	HA head	Neutralization of H1N1 strains	MDCK cells	[110]
F045–092	HA head	Neutralization to the entire H3 subtype	10-day-old Embryonated chicken eggs	[50]
H3v-47	HA head	Neutralization of various H3N2 strains; Protection against an H3N2 strain after intraperitoneal injection	MDCK cells; Mouse	[121]
KL-H4–1E8, KL-H4–1G4, KL-H4–2B1, KL-H4–3D8, KL-H4–3G7, KL-H4–4A11, KL-H4–4E8, KL-H4–5B8	HA head	Binding to various H4 HA; Protection against an H4N6 strain after intraperitoneal injection	Mouse	[122]
CR6261	HA stalk	Neutralization to H1, H2, H5, H6, H8, and H9 strains; Protection against H1N1 and H5N1 strains after intraperitoneal injection	MDCK cells; Mouse	[132]
F10	HA stalk	Inhibition to cell fusion; Protection against H5 strains after intraperitoneal injection	MDCK cells; HeLa cells; Mouse	[145]
FI6	HA stalk	Neutralization to group I and II strains	MDCK cells	[114]
CR8020	HA stalk	Protection against H3N2 and H7N7 strains	Mouse	[146]
6F12	HA stalk	Neutralization to H1 strains; Protection against H1 strains after intraperitoneal injection or passive immunization	MDCK cells; Mouse; Ferret	[138,147]
39.29	HA stalk	Neutralization to H1, H2, and H3 strains; Protection against H1N1, H3N2 and H5N1 strains	MDCK cells; Mouse; Ferret	[119]
CR8043	HA stalk	Neutralization to H3 and H10 strains; Protection against H3N2 and H7N7 strains.	MDCK cells; Mouse	[139]
MEDI8852	HA stalk	Neutralization to group I and II strains; Protection against H1, H3, and H5 strains after intraperitoneal infection or intranasal immunization.	MDCK cells; Mouse; Ferret	[141]
27F3	HA stalk	Binding to H1, H2, H3, H5, H6, H7, H9, H11, H12, H13, H16, and Flu B strains	-	[133]

In addition, selected stalk domains were used for the development of a universal vaccine [41,148]. Self-assembling ferritin nanoparticles displaying HA stalk trimers could be recognized by broadly neutralizing antibodies (bNAbs) [149]. A linear neutralizing epitope locating in the C terminus of the helix A region in H7N9 HA was discovered and identified as a novel linear cross-reactive epitope contributing to the development of a universal vaccine [150]. Therefore, although current inactivated influenza vaccines induce minimal levels of HA stalk antibodies [151], emerging strategies were designed to focus on the highly conserved stalk region among the different subtypes of IAVs.

3.2. Universal Vaccines against the Hemagglutinin

Vaccines, including inactivated or live attenuated vaccines, are considered the most efficacious and cost-effective measure to provide protection against AIV. The former approach can elicit an antibody response, whereas the latter elicits a broader response [152]. Since the HA protein presents a high plasticity in sequence, a small number of mutations in the HA gene could result in a significant reduction in vaccine efficiency [153,154]. Cluster-transition substitutions provide clues of the underlying mechanism. It was suggested that single amino acid substitutions or combinations of a few substitutions contribute to antigenic drift [40]. Recently, based on machine learning, a novel computational method named RECDS (recognition of cluster-transition determining sites) was developed. The method jointly characterizes information of the sequences as well as the antigenic evolution of the viruses. On this basis, it recognized 10–15 critical cluster-transition substitutions in H3N1 and H1N1. This method could be applied for other influenza viruses and potentially help the vaccine strain selection [155].

It is generally accepted that the efficacy of current traditional inactivated vaccines has been limited to how well the vaccine strains and circulating strains are matched. Thus, current seasonal vaccines require frequent updating of the vaccine formulation. Therefore, universal influenza vaccines which can elicit broad cellular or/and humoral responses against variant AIV strains have become a global concern. Furthermore, a mathematical model analyzing the interaction between vaccination and viral evolution suggests that universal vaccines are more efficient than conventional vaccines in annual influenza epidemic control [156].

A criterion of a universal vaccine defined by National Institute of Allergy and Infectious Disease (NIAID) is that the vaccine should provide ≥75% efficacy against seasonal flu and a broad protection against symptomatic group I and II influenza infections and the protection could persist for at least one year. Since the HA protein serves as the predominant antigenic protein of the virus, the structural and functional characterization of HA has provided novel insights into vaccine design and therapeutic methods against the virus. For instance, immune complexes (ICs) comprising the seasonal influenza vaccine (TIV) and broadly reactive Fc anti-HA IgGs have been shown to enhance a protective response for breadth [157]. Alphavirus-vectored HA subunit vaccines, delivering a monovalent or bivalent HA, have been tested to be able to provide efficient homo/heterosubtypic protection [158]. A plasmid fused with anti-head peptide (NG34) and cytotoxic T lymphocyte-associated antigen (CTLA4) has been shown to be protective against the heterosubtypic H3N2 virus in swine [159]. Furthermore, for efficient HA-based vaccine design, complexities such as the efficiency of HA epitopes and antibody interference, should be taken into consideration [160–162].

3.2.1. HA Stalk-Based Universal Vaccines

Due to the plasticity of the HA head domain, though antibodies against this region are often long-lived [163], they can become ineffective through antigenic changes in the head region [164,165]. Based on the evolutionary and selection analysis of the stalk domain, researchers suggest that the stalk domain evolves at a slower rate than the head domain and may not be directly responsible for escaping antibody neutralization [166].

Since several broadly neutralizing antibodies, such as CR6261 and F10 [132,145], were found to bind specifically to the HA stalk region, the stalk region further became of major interest as a target for the design of universal vaccines. A display-platform bearing heterosubtypic HA stalk peptides were developed in order to select identified stalk sequences with protective efficacy [167]. To elicit HA stalk-specific immune responses, several approaches were developed. For instance, mild low-temperature treatment (at ≤25 °C) could moderately change the structure of HA, thus inducing an enhanced stalk specific antibody response which results in cross-protection in mice [168]. Heterologous prime-boost immunization could also elicit HA stalk-specific antibodies [169,170]. Investigation on the immunogenicity of glycol-forms of the stalk region suggests that glycosylation plays an important role in providing broad protection in mice [171].

One thing that needs to be taken into consideration is that the immunodominance of the HA head may lower the efficiency of the stem vaccines. Generally, to prevent the interference, there are two strategies to induce stalk-based immunity: headless HA constructs and chimeric HAs (cHAs)

composed of conserved stalks and mismatched heads [172–177]. For the "headless" approach, in both vaccinated mice and monkeys, "headless" HA could produce levels of anti-stalk broadly neutralizing antibodies and protect the animals from homo-/heterosubtypic challenge [114,172,178–181]. H1 HA stalk nanoparticles generated by six iterative cycles of structure-based design could elicit broadly cross-reactive antibodies in mice and ferrets [182]. When an H5 or H1 "headless" HA was vaccinated to mice, the vaccinated animals were protected against lethal challenge with group 1 IAVs [173,183]. Since the long α-helix (LAH) at the membrane distal part of the stalk domain is usually covered by the head domain, removing the head domain would destabilize the stalk region. A helical leucine zipper trimerization domain was applied to stabilize the headless construct and induce stalk-specific antibodies with protection against highly pathogenic H5N1 viruses in animal models [180].

The second scenario applies chimeric HAs in order to break the immunodominance of the head domain. Several researchers found that sequential vaccination with the same HA stalk but divergent HA heads could induce stalk-specific antibodies, resulting in protection against heterologous challenge [174,175]. Therefore, in this approach, the subdominant stalk domain is considered to be re-targeted by the immune response. In mice and ferret models, this concept was proven to provide protection against heterosubtypic challenges [138,174,177,184,185]. Recently, in mice, the enhanced protection by chimeric live attenuated influenza vaccines was shown to be driven by stalk reactive IgG antibodies, comparing to the relevant viruses expressing natural HAs [186].

Considering the pre-existing immunity in human populations, which leads to an inhibition of vaccine-induced immunity to the stalk region [187], whether clinically the administration of cHA vaccines would be sufficient enough remains to be further tested, and the careful selection of both the HA head and stalk epitopes may help the vaccine efficiency [157,131].

3.2.2. HA TM-Domain-Based Universal Vaccines

The transmembrane (TM) domain is composed of 25–28 amino acids [188], which are responsible for retaining the homotrimer HA on the viral membrane. Several mutations in this region were demonstrated to be able to affect viral fusion [189], replication [189,190], infection [191,192] and HA distribution [193]. The TM domain mainly contains hydrophobic residues. Whether the absence of this region could affect the trimerization of the HA conformation is still controversial and future work is required [43,194]. Researchers found that the stability and immunogenicity of full-length HA were better than HA lacking the TM domain in the mouse model. The H7 HA containing only the ectodomain could not induce robust HI and neutralizing antibody titers in mice [195].

Apart from the other 17 HA subtypes, the H3 HA contains two conserved cysteines (540Cys, 544Cys) locating in the TM domain. Our lab found that mutations of the two cysteines to 540Ser and 544Leu could diminish the thermal stability and the enhance fusion activity of the H3 HA [88]. Substitutions of the two cysteines or substitution of the H3 HA TM domain could regulate the fusion activity of H3N2 viruses [89]. The underlying mechanism might be that the disulfide bond between the two cysteines could enhance the stability of the HA trimers [91]. By introducing a CFLLC mini-domain into the TM domain of H1, H5, H7 and H9 HA TM domains, the modified HAs presented their cross-reactivity and cross-protection over the wild-type HAs [43,88,196,197]. These results suggest the importance of the TM domain in HA structural stability as well as viral biological characteristics.

When immunized in mice, the substitution of the TM region in H1, H3, H5 and H9 with H3 TM domains could induce increased antibody and cytokine responses [43]. Furthermore, the TM-replaced HA of H1, H5 and H9 all presented higher cross immunogenicity than the wild types, suggesting a possible way to induce hetero-protection with the H3-TM replaced strategy [43,196]. To evaluate the TM-replacement strategy in the rescued viruses, our lab further constituted TM-substituted H7N9 and H9N2 recombinant viruses applying reverse genetics [198,199]. The strategy of TM-replacement resulted in a higher ability in forming HA trimers, better structural stability under extreme temperature or pH conditions compared to the wild type. The inactivated vaccines using these TM-replaced viruses could induce higher HI titers, HA-specific antibody titers and complete protection against homologous or heterologous H7N2 or H9N2 strains [198,199]. Moreover, the

recombinant H7N9 virus could induce higher IFN-γ levels against HA subtypes of different branches [198]. Virus-like particles (VLPs) containing H3-TM replaced HA of H5 and H7 subtypes induced enhanced antibody responses and higher IFN-γ [200,201]. In mice, the H7 VLPs-TM could provide better protection against homologous and heterologous H7N9 viruses [200]. These results suggest that TM-replacement vaccines could provide protection efficiency in a broader way. A summary of the strategies for HA-based universal vaccine development mentioned in this review is presented in Figure 2.

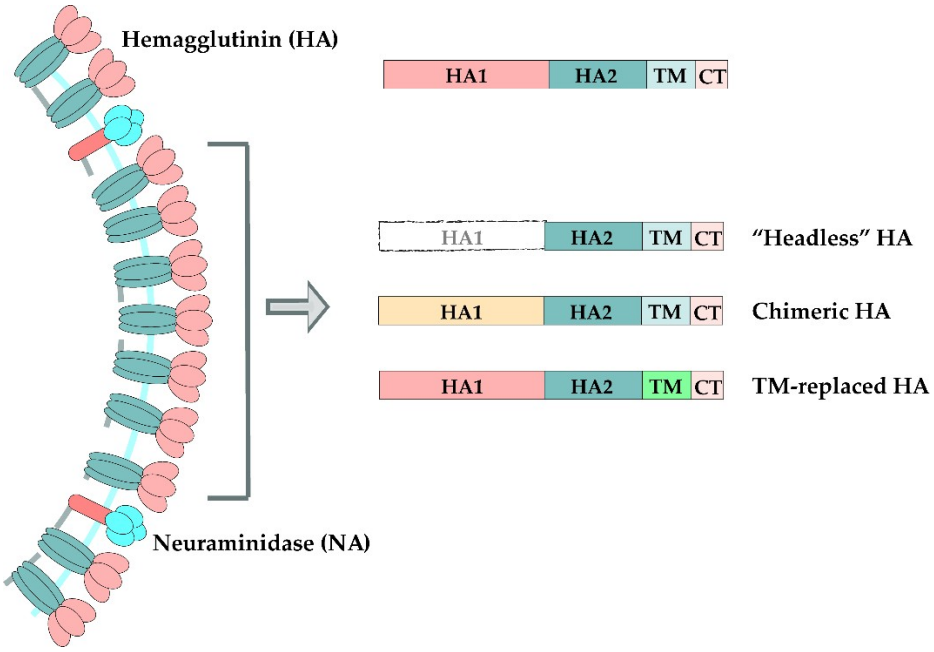

Figure 2. The schematic diagram of various strategies in the universal influenza vaccine development.

3.3. HA Inhibitors as Anti-Influenza Drugs

The available anti-influenza drugs are, so far, M2 ion channel blockers (amantadine and rimantadine) and NA inhibitors (peramivir, laninamivir, zanamivir, and oseltamivir) [25,202–204]. However, due to the continually emerging M2 or NA inhibitor resistance, the clinical application of these inhibitors is limited now [205,206]. In contrast to the investigation on M2 and NA inhibitors, the searches for HA inhibitors as anti-influenza drugs have been challenging. To block the entry of the IAVs into the host cell, one plausible target is the sialic acid binding (SAB) site on HA since the SAB site is relatively conserved [207].

One promising approach for searching HA inhibitors is to develop an inhibitor that will block the fusion of HA with endosome. Several compounds have been suggested to function as fusion inhibitors [72]. Compounds such as CL-61917, CL-385319, and CL-62554 [208], BMY-27709 [209], LY-180299 [210], RO5464466 and RO5487624 [211], FA-583 and FA-617 [212] target group I HAs, whereas TBHQ [213,10], S19 and C22 [214] are fusion inhibitors to group II HAs. Recently, peptides such as PEP87 were found to be able to bind to the HA trimmer and thus disrupted HA-mediated entry [215]. Arbidol was found to inhibit HA for both group I and group II HAs by binding to a hydrophobic cavity in the stem region [216,217]. Utilizing the structural details of HA stem broadly neutralizing antibody (bnAb CR6261), a small-molecule inhibitor JNJ4796 was optimized and showed to be orally active in mice [218]. A summary of the HA inhibitors mentioned in this review is presented in Table 2.

Table 2. The summary of anti-HA drugs.

Drugs	Target	Function	Animal/Cell	Reference

BMY-27709	HA2	Inhibit replication of the H1 and H2 viruses in the early stage	MDBK cells	[209]
C22	HA2	Facilitate the HA conformational change; Inhibit fusion activity and viral infection	MDCK cells	[214]
LY-180299	HA	Inhibits membrane fusion; Inhibit replication of H1N1 virus in the early stage	MDCK cells	[210]
CL-61917, CL-385319, CL-62554	HA2	Inhibit viral replication; Inhibit virus-specific protein synthesis; Inhibit cell-to-cell fusion	MDCK cells	[208]
TBHQ	HA2	Inhibit viral infectivity of H3 strains; Stabilize the HA neutral pH structure; Inhibit membrane fusion	MDCK cells	[213,10]
Arbidol	HA stalk	Bind in a hydrophobic cavity in the HA stalk region; Inhibit early membrane fusion and viral replication; Stabilize HA pre-fusion conformation	MDCK cells	[216,217]
RO5464466, RO5487624	HA	Inhibit viral replication in the early stage; Block fusion by stabilizing the HA pre-fusion structure; Protection against H1N1 strain by intravenous administration	MDCK cells; Mouse	[211]
FA-583, FA-617	HA2	Inhibit the fusion of group I HA; Prohibit low-pH-induced HA conformational change	MDCK cells	[212]
PEP87	HA2	Inhibit H7 and H5 HA-mediated entry; Disrupt the HA pre-fusion structure	HEK 293T cells	[215]
JNJ4796	HA stalk	Neutralize group I viruses, Inhibit HA-mediated fusion; Protection against H1N1 strain after oral administration	MDCK cells; Mouse; HBECs	[218]

Considering the high variability and rapid microevolution of the virus, searching for new antiviral agents, especially HA inhibitors, is required [219,220]. Techniques such as deep sequencing and hydrogen-deuterium exchange mass spectrometry (HDX-MS) have been applied for epitope mapping to search for potential drug candidates [37,221,222]. Furthermore, since the HA protein is not an enzyme, the inhibition to HA trimerization, pH-induced conformational transition, or HA-induced membrane fusion should be taken into consideration for anti-HA drug design and a suitable in vitro test system should be carefully chosen.

4. Perspectives

The concept of "universal" vaccine is to cover a large subset of influenza viruses independent of antigenic drift. Considering the dynamic epidemiology and genetic diversity of the virus, targeting a highly conserved antigenic domain would be an effective way for universal IAV vaccine development. Different methods including the transformation of the HA protein as well as NP, PA, and M1 proteins have been considered to provide cross-protection against heterosubtypic challenges. In addition, vaccination methods and strategies would be also important.

Since current traditional inactivated vaccines do not stimulate cellular immune responses, novel technologies to stimulate T cell immunity were tested [223]. To avoid the activation of strain-specific B cells, a mosaic array by co-localizing heterotypic RBDs on a single nanoparticle was designed to promote cross-reactive antibody responses [224]. The use of adjuvants, such as nano-emulsion adjuvant NE_{01} [225] and synthetic adjuvant SF_{-10} [226] etc., were also applied to improve the breadth of influenza vaccine coverage. The computational method was also applied in the HA-based strategy

for "universal" vaccine development. Take Computational Optimized Broadly Reactive Antigen (COBRA) technology for instance, the method uses consensus sequences to design broadly reactive influenza immunogens [227–231].

Overall, multiple strategies were applied in order to generate universal and cross-protective influenza vaccines. However, since researchers also showed that the cross-protection provided by the universal IAV vaccine was weak [232] and sometimes accompanied by exacerbated clinical signs (in swine) [233], the performance of the updated universal influenza vaccines is required to be tested over multiple influenza seasons, and the careful clinical application of the universal vaccine is also suggested.

Author Contributions: Writing – Original Draft Preparation, Y.Z. and C.X.; Writing – Review & Editing, G.L. and H.Z.; Supervision, Y.C.; Funding Acquisition, Y.Z. and Y.C. All authors read and approved the final version of the manuscript.

Funding: This study was supported by the "Zhujiang talent program" overseas youth talent introduction program (post-doctoral program), the open project of the State Key Laboratory of Biocontrol (SKLBC16KF01) and Doctoral Initiative Project of Natural Science Foundation of Guangdong Province (20183300031680007).

Conflicts of Interest: The authors declare that they have no financial and personal relationships with other people or organizations that can influence the work. There is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in this review. They do not have any commercial or associative interest that represents conflicts of interest in connection with the work submitted.

References

1. World Health Organization (2018) Influenza (Seasonal.) Available online: www.who.int/en/news-room/fact-sheets/detail/influenza-(seasonal) (accessed 6 December 2018).
2. Wagner, R.; Matrosovich, M.; Klenk, H.D. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. *Rev. Med. Virol.* **2002**, *12*, 159–166.
3. Heaton, N.S.; Sachs, D.; Chen, C.J.; Hai, R.; Palese, P. Genome-wide mutagenesis of influenza virus reveals unique plasticity of the hemagglutinin and NS1 proteins. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 20248–20253.
4. Gamblin, S.J.; Skehel, J.J. Influenza hemagglutinin and neuraminidase membrane glycoproteins. *J. Biol. Chem.* **2010**, *285*, 28403–28409.
5. Yang, J.; Li, M.; Shen, X.; Liu, S. Influenza A virus entry inhibitors targeting the hemagglutinin. *Viruses* **2013**, *5*, 352–373.
6. Bailey, E.S.; Choi, J.Y.; Fieldhouse, J.K.; Borkenhagen, L.K.; Zemke, J.; Zhang, D.; Gray, G.C. The continual threat of influenza virus infections at the human-animal interface: What is new from a one health perspective? *Evol. Med. Public Health* **2018**, *2018*, 192–198.
7. Mineev, K.S.; Lyukmanova, E.N.; Krabben, L.; Serebryakova, M.V.; Shulepko, M.A.; Arseniev, A.S.; Kordyukova, L.V.; Veit, M. Structural investigation of influenza virus hemagglutinin membrane-anchoring peptide. *Protein Eng. Des. Sel.* **2013**, *26*, 547–552.
8. Pica, N.; Palese, P. Toward a universal influenza virus vaccine: Prospects and challenges. *Annu. Rev. Med.* **2013**, *64*, 189–202.
9. Air, G.M. Sequence relationships among the hemagglutinin genes of 12 subtypes of influenza A virus. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 7639–7643.
10. Russell, R.J.; Kerry, P.S.; Stevens, D.J.; Steinhauer, D.A.; Martin, S.R.; Gamblin, S.J.; Skehel, J.J. Structure of influenza hemagglutinin in complex with an inhibitor of membrane fusion. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 17736–17741.
11. Kordyukova, L.V.; Serebryakova, M.V.; Polyansky, A.A.; Kropotkina, E.A.; Alexeevski, A.V.; Veit, M.; Efremov, R.G.; Filippova, I.Y.; Baratova, L.A. Linker and/or transmembrane regions of influenza A/Group-1, A/Group-2, and type B virus hemagglutinins are packed differently within trimers. *Biochim. Biophys. Acta* **2011**, *1808*, 1843–1854.
12. Tong, S.; Zhu, X.; Li, Y.; Shi, M.; Zhang, J.; Bourgeois, M.; Yang, H.; Chen, X.; Recuenco, S.; Gomez, J.; et al. New world bats harbor diverse influenza A viruses. *PLoS Pathog.* **2013**, *9*, e1003657.
13. Alexander, D.J. An overview of the epidemiology of avian influenza. *Vaccine* **2007**, *25*, 5637–5644.
14. Munster, V.J.; Schrauwen, E.J.; de Wit, E.; van den Brand, J.M.; Bestebroer, T.M.; Herfst, S.; Rimmelzwaan, G.F.; Osterhaus, A.D.; Fouchier, R.A. Insertion of a multibasic cleavage motif into the hemagglutinin of a low-pathogenic avian influenza H6N1 virus induces a highly pathogenic phenotype. *J. Virol.* **2010**, *84*, 7953–7960.

15. Soda, K.; Asakura, S.; Okamatsu, M.; Sakoda, Y.; Kida, H. H9N2 influenza virus acquires intravenous pathogenicity on the introduction of a pair of di-basic amino acid residues at the cleavage site of the hemagglutinin and consecutive passages in chickens. *Virol. J.* **2011**, *8*, 64.
16. Veits, J.; Weber, S.; Stech, O.; Breithaupt, A.; Gräber, M.; Gohrbandt, S.; Bogs, J.; Hundt, J.; Teifke, J.P.; Mettenleiter, T.C.; et al. Avian influenza virus hemagglutinins H2, H4, H8 and H14 support a highly pathogenic phenotype. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 2579–2584.
17. Zhang, F.; Bi, Y.; Wang, J.; Wong, G.; Shi, W.; Hu, F.; Yang, Y.; Yang, L.; Deng, X.; Jiang, S.; et al. Human infections with recently-emerging highly pathogenic H7N9 avian influenza virus in China. *J. Infect.* **2017**, *75*, 71–75.
18. Ungchusak, K.; Auewarakul, P.; Dowell, S.F.; Kitphati, R.; Auwanit, W.; Puthavathana, P.; Uiprasertkul, M.; Boonnak, K.; Pittayawonganon, C.; Cox, N.J.; et al. Probable person-to-person transmission of avian influenza a (H5N1). *N. Engl. J. Med.* **2005**, *352*, 333–340.
19. Lai, S.; Qin, Y.; Cowling, B.J.; Ren, X.; Wardrop, N.A.; Gilbert, M.; Tsang, T.K.; Wu, P.; Feng, L.; Jiang, H.; et al. Global epidemiology of avian influenza A H5N1 virus infection in humans, 1997–2015: A systematic review of individual case data. *Lancet Infect. Dis.* **2016**, *16*, e108–e118.
20. Jiang, H.; Wu, P.; Uyeki, U.M.; He, J.; Deng, Z.; Xu, W.; Lv, Q.; Zhang, J.; Wu, Y.; Tsang, T.K.; et al. Preliminary epidemiologic assessment of human infections with highly pathogenic avian influenza A (H5H6) virus, China. *Clin. Infect. Dis.* **2017**, *65*, 383–388.
21. Iuliano, A.D.; Jang, Y.; Jones, J.; Davis, C.T.; Wentworth, D.E.; Uyeki, T.M.; Roguski, K.; Thompson, M.G.; Gubareva, L.; Fry, A.M.; et al. Increase in human infections with avian influenza A (H7N9) virus during the fifth epidemic – China, October 2016-February 2017. *MMWR Morb. Mortal. Wkly. Rep.* **2017**, *66*, 254–255.
22. Sun, Y.; Qin, K.; Wang, J.; Pu, J.; Tang, Q.; Hu, Y.; Bi, Y.; Zhao, X.; Yang, H.; Shu, Y.; et al. High genetic compatibility and increased pathogenicity of reassortants derived from avian H9N2 and pandemic H1N1/2009 influenza viruses. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 4164–4169.
23. Zhang, Y.; Zhang, Q.; Kong, Y.; Jiang, Y.; Gao, Y.; Deng, G.; Shi, J.; Tian, G.; Liu, L.; Liu, J.; et al. H5N1 hybrid viruses bearing 2009/H1N1 virus genes transmit in guinea pigs by respiratory droplet. *Science* **2013**, *340*, 1459–1463.
24. Li, X.; Liu, B.; Ma, S.; Cui, P.; Liu, W.; Li, Y.; Guo, J.; Chen, H. High frequency of reassortment after co-infection of chicken with the H4N6 and H9N2 influenza A viruses and the biological characteristics of the reassortants. *Vet. Microbiol.* **2018**, *222*, 11–17.
25. Zeng, L.Y.; Yang, J.; Liu, S. Investigational hemagglutinin-targeted influenza virus inhibitors. *Expert Opin. Investig. Drugs* **2017**, *26*, 63–73.
26. Carrat, F.; Flahault, A. Influenza vaccine: The challenge of antigenic drift. *Vaccine* **2007**, *25*, 6852–6862.
27. Kilbourne, E.D. Influenza pandemics of the 20th century. *Emerg. Infect. Dis.* **2006**, *12*, 9–14.
28. Bui, C.M.; Chughtai, A.A.; Adam, D.C.; MacIntyre, C.R. An overview of the epidemiology and emergence of influenza A infection in humans over time. *Arch. Public Health* **2017**, *75*, 15.
29. Osterholm, M.T.; Kelly, N.S.; Sommer, S.; Belongia, E.A. Efficacy and effectiveness of influenza vaccines: A systematic review and meta-analysis. *Lancet Infect. Dis* **2012**, *12*, 36–44.
30. Kargarfard, F.; Sami, A.; Hemmatzadeh, F.; Ebrahimie, E. Identifying mutation positions in all segments of influenza genome enables better differentiation between pandemic and seasonal strains. *Gene* **2019**, *697*, 78–85.
31. Mancini, N.; Solforosi, L.; Clementi, N.; de Marco, D.; Clementi, M.; Burioni, R. A potential role for monoclonal antibodies in prophylactic and therapeutic treatment of influenza. *Antiviral. Res.* **2011**, *92*, 15–26.
32. Bhatt, S.; Holmes, E.C.; Pybus, O.G. The genomic rate of molecular adaptation of the human influenza A virus. *Mol. Biol. Evol.* **2011**, *28*, 2443–2451.
33. Nobusawa, E.; Aoyama, T.; Kato, H.; Suzuki, Y.; Tateno, Y.; Nakajima, K. Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. *Virology* **1991**, *182*, 475–485.
34. Tzarum, N.; de Vries, R.P.; Peng, W.; Thompson, A.J.; Bouwman, K.M.; McBride, R.; Yu, W.; Zhu, X.; Verheije, M.H.; Paulson, J.C.; et al. The 150-loop restricts the host specificity of human H10N8 influenza virus. *Cell Rep.* **2017**, *19*, 235–245.
35. Velkov, T.; Ong, C.; Baker, M.A.; Kim, H.; Li, J.; Nation, R.L.; Huang, J.X.; Cooper, M.A.; Rockman, S. The antigenic architecture of the hemagglutinin of influenza H5N1 viruses. *Mol. Immunol.* **2013**, *56*, 705–719.
36. Thyagarajan, B.; Bloom, J.D. The inherent mutational tolerance and antigenic evolvability of influenza hemagglutinin. *eLife* **2014**, *3*, e03300.
37. Wu, N.C.; Young, A.P.; Al-Mawsawi, L.Q.; Olson, C.A.; Feng, J.; Qi, H.; Chen, S.H.; Lu, I.H.; Lin, C.Y.; Chin, R.G.; et al. High-throughput profiling of influenza A virus hemagglutinin gene at single-nucleotide resolution. *Sci. Rep.* **2014**, *4*, 4942.

38. Wu, N.C.; Thompson, A.J.; Xie, J.; Lin, C.W.; Nycholat, C.M.; Zhu, X.; Lerner, R.A.; Paulson, J.C.; Wilson, I.A. A complex epistatic network limits the mutational reversibility in the influenza hemagglutinin receptor-binding site. *Nat. Commun.* **2018**, *9*, 1264.
39. Cao, Y.; Koh, X.; Dong, L.; Du, X.; Wu, A.; Ding, X.; Deng, H.; Shu, Y.; Chen, J.; Jiang, T. Rapid estimation of binding activity of influenza virus hemagglutinin to human and avian receptors. *PLoS ONE* **2011**, *6*, e18664.
40. Koel, B.F.; Burke, D.F.; Bestebroer, T.M.; van der Vliet, S.; Zondag, G.C.; Vervaet, G.; Skepner, E.; Lewis, N.S.; Spronken, M.I.; Russell, C.A.; et al. Substitutions near the receptor binding site determine major antigenic change during influenza virus evolution. *Science* **2013**, *342*, 976–979.
41. Wang, T.T.; Tan, G.S.; Hai, R.; Pica, N.; Ngai, L.; Ekiert, D.C.; Wilson, I.A.; García-Sastre, A.; Moran, T.M.; Palese, P. Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 18979–18984.
42. Chen, S.; Zheng, D.; Li, C.; Zhang, W.; Xu, W.; Liu, X.; Fang, F.; Chen, Z. Protection against multiple subtypes of influenza viruses by virus-like particle vaccines based on a hemagglutinin conserved epitope. *Biomed. Res. Int.* **2015**, *2015*, 901817.
43. Liu, Q.; Liu, K.; Xue, C.; Zhou, J.; Li, X.; Luo, D.; Zheng, J.; Xu, S.; Liu, G.D.; Cao, Y. Recombinant influenza H1, H5 and H9 hemagglutinins containing replaced H3 hemagglutinin transmembrane domain showed enhanced heterosubtypic protection in mice. *Vaccine* **2014**, *32*, 3041–3049.
44. Stray, S.J.; Pittman, L.B. Subtype- and antigenic site-specific differences in biophysical influences on evolution of influenza virus hemagglutinin. *Virol. J.* **2012**, *9*, 91.
45. Wang, X.; Ilyushina, N.A.; Lugovtsev, V.Y.; Bovin, N.V.; Couzens, L.K.; Gao, J.; Donnelly, R.P.; Eichelberger, M.C.; Wan, H. Amino acids in hemagglutinin antigenic site B determine antigenic and receptor binding differences between A(H3N2)v and ancestral seasonal H3N2 influenza viruses. *J. Virol.* **2017**, *91*, e01512–e01516.
46. Cueno, M.E.; Shiotsu, H.; Nakano, K.; Sugiyama, E.; Kikuta, M.; Usui, R.; Oya, R.; Imai, K. Structural significance of residues 158–160 in the H3N2 hemagglutnin globular head: A computational study with implications in viral evolution and infection. *J. Mol. Graph. Model.* **2019**, *89*, 33–40.
47. Guarnaccia, T.; Carolan, L.A.; Maurer-Stroh, S.; Lee, R..; Job, E.; Reading, P.C.; Petrie, S.; McCaw, J.M.; McVernon, J.; Hurt, A.C.; et al. Antigenic drift of the pandemic 2009 A(H1N1) influenza virus in A ferret model. *PLoS Pathog.* **2013**, *9*, e1003354.
48. Li, C.; Hatta, M.; Burke, D.F.; Ping, J.; Zhang, Y.; Ozawa, M.; Taft, A.S.; Das, S.C.; Hanson, A.P.; Song, J.; et al. Selection of antigenically advanced variants of seasonal influenza viruses. *Nat. Microbiol.* **2016**, *1*, 16058.
49. Igarashi, M.; Ito, K.; Kida, H.; Takada, A. Genetically destined potentials for N-linked glycosylation of influenza virus hemagglutinin. *Virology* **2008**, *376*, 323–329.
50. Lee, P.S.; Ohshima, N.; Stanfield, R.L.; Yu, W.; Iba, Y.; Okuno, Y.; Kurosawa, Y.; Wilson, I.A. Receptor mimicry by antibody F045–092 facilitates universal binding to the H3 subtype of influenza virus. *Nat. Commun.* **2014**, *5*, 3614.
51. Tate, M.D.; Job, E.R.; Deng, Y.M.; Gunalan, V.; Maurer-Stroh, S.; Reading, P.C. Playing hide and seek: How glycosylation of the influenza virus hemagglutinin can modulate the immune response to infection. *Viruses* **2014**, *6*, 1294–1316.
52. Das, S.R.; Hensley, S.E.; David, A.; Schmidt, L.; Gibbs, J.S.; Puigbò, P.; Ince, W.L.; Bennink, J.R.; Yewdell, J.W. Fitness costs limit influenza A virus hemagglutinin glycosylation as an immune evasion strategy. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, E1417–E1422.
53. Kim, J.I.; Lee, I.; Park, S.; Hwang, M.W.; Bae, J.Y.; Lee, S.; Heo, J.; Park, M.S.; García-Sastre, A.; Park, M.S. Genetic requirement for hemagglutinin glycosylation and its implications for influenza A H1N1 virus evolution. *J. Virol.* **2013**, *87*, 7539–7549.
54. Alymova, I.V.; York, I.A.; Air, G.M.; Cipollo, J.F.; Gulati, S.; Baranovich, T.; Kumar, A.; Zeng, H.; Gansebom, S.; McCullers, J.A. Glycosylation changes in the globular head of H3N2 influenza hemagglutinin modulate receptor binding without affecting virus virulence. *Sci. Rep.* **2016**, *6*, 36216.
55. Vachieri, S.G.; Xiong, X.; Collins, P.J.; Walker, P.A.; Martin, S.R.; Haire, L.F.; Zhang, Y.; McCauley, J.W.; Gamblin, S.J.; Skehel, J.J. Receptor binding by H10 influenza viruses. *Nature* **2014**, *511*, 475–477.
56. Carvalho, S.B.; Moleirinho, M.G.; Wheatley, D.; Welsh, J.; Gantier, R.; Alves, P.M.; Peixoto, C.; Carrondo, M.J.T. Universal label-free in-process quantification of influenza virus-like particles. *Biotechnol. J.* **2017**, *12*, 1700031.
57. Gooding, J.J.; Parker, S.G.; Lu, Y.; Gaus, K. Molecularly engineered surfaces for cell biology: From static to dynamic surfaces. *Langmuir* **2014**, *30*, 3290–3302.
58. Satav, T.; Huskens, J.; Jonkheijm, P. Effects of variations in ligand density on cell signaling. *Small* **2015**, *11*, 5184–5199.
59. Koçer, G.; Jonkheijm, P. Guiding hMSC Adhesion and Differentiation on Supported Lipid Bilayers. *Adv. Healthc. Mater.* **2017**, *6*, doi:10.1002/adhm.201600862.

60. Di lorio, D.; Verheijden, M.L.; van der Vries, E.; Jonkheijm, P.; Huskens, J. Weak multivalent binding of influenza hemagglutinin nanoparticles at a sialoglycan-functionalized supported lipid bilayer. *ACS Nano* **2019**, *13*, 3413–3423.
61. Liu, J.; Stevens, D.J.; Haire, L.F.; Walker, P.A.; Coombs, P.J.; Russell, R.J.; Gamblin, S.J.; Skehel, J.J. Structures of receptor complexes formed by hemagglutinins from the asian influenza pandemic of 1957. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 17175–17180.
62. Shelton, H.; Ayora-Talavera, G.; Ren, J.; Loureiro, S.; Pickles, R.J.; Barclay, W.S.; Jones, I.M. Receptor binding profiles of avian influenza virus hemagglutinin subtypes on human cells as a predictor of pandemic potential. *J. Virol.* **2011**, *85*, 1875–1880.
63. Franca, M.; Stallknecht, D.E.; Howerth, E.W. Expression and distribution of sialic acid influenza virus receptors in wild birds. *Avian Pathol.* **2013**, *42*, 60–71.
64. De Graaf, M.; Fouchier, R.A. Role of receptor binding specificity in influenza A virus transmission and pathogenesis. *EMBO J.* **2014**, *33*, 823–841.
65. Nicholls, J.M.; Chan, R.W.; Russell, R.J.; Air, G.M.; Peiris, J.S. Evolving complexities of influenza virus and its receptors. *Trends Microbiol.* **2008**, *16*, 149–157.
66. Stray, S.J.; Cumming, R.D.; Air, G.M. Influenza virus infection of desialylated cells. *Glycobiology* **2000**, *10*, 649–658.
67. Zhao, N.; Martin, B.E.; Yang, C.K.; Luo, F.; Wan, X.F. Association analyses of large-scale glycan microarray data reveal novel host-specific substructures in influenza A virus binding glycans. *Sci. Rep.* **2015**, *5*, 15778.
68. Chaipan, C.; Kobasa, D.; Bertram, S.; Glowacka, I.; Steffen, I.; Tsegaye, T.S.; Takeda, M.; Bugge, T.H.; Kim, S.; Park, Y.; et al. Proteolytic activation of the 1918 influenza virus hemagglutinin. *J. Virol.* **2009**, *83*, 3200–3211.
69. Bottcher, E.; Matrosovich, T.; Beyerle, M.; Klenk, H.D.; Garten, W.; Matrosovich, M. Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium. *J. Virol.* **2006**, *80*, 9896–9898.
70. Steiner, D.F.; Smeekens, S.P.; Ohagi, S.; Chan, S.J. The new enzymology of precursor processing endoproteases. *J. Biol. Chem.* **1992**, *267*, 23435–23438.
71. Stieneke-Grober, A.; Vey, M.; Angliker, H.; Shaw, E.; Thomas, G.; Roberts, C.; Klenk, H.D.; Garten, W. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *EMBO J.* **1992**, *11*, 2407–2414.
72. Xu, R.; Wilson, I.A. Structural characterization of an early fusion intermediate of influenza virus hemagglutinin. *J. Virol.* **2011**, *85*, 5172–5182.
73. Luo, M. Influenza virus entry. *Adv. Exp. Med. Biol.* **2012**, *726*, 201–221.
74. Smrt, S.T.; Lorieau, J.L. Membrane fusion and infection of the influenza hemagglutinin. *Adv. Exp. Med. Biol.* **2017**, *966*, 37–54.
75. Worch, R.; Krupa, J.; Filipek, A.; Szymaniec, A.; Setny, P. Three conserved C-terminal residues of influenza fusion peptide alter its behavior at the membrane interface. *Biochim. Biophys. Acta* **2017**, *1861*, 97–105.
76. Chen, J.; Skehel, J.J.; Wiley, D.C. N- and C-terminal residues combine in the fusion-pH influenza hemagglutinin HA(2) subunit to form an N cap that terminates the triple-stranded coiled coil. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 8967–8972.
77. Di Lella, S.; Herrmann,A.; Mair, C.M. Modulation of the pH stability of influenza virus hemagglutinin: A host cell adaption strategy. *Biophys J.* **2016**, *110*, 2293–2301.
78. Russier, M.; Yang, G.; Rehg, J.E.; Wong, S.S.; Mostafa, H.H.; Fabrizio, T.P.; Barman, S.; Krauss, S.; Webster, R.G.; Webby, R.J.; et al. Molecular requirements for a pandemic influenza virus: An acistable hemagglutinin protein. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 1636–1641.
79. Wu, N.C.; Wilson, I.A. A perspective on the structural and functional constraints for immune evasion: Insights from influenza virus. *J. Mol. Biol.* **2017**, *429*, 2694–2709.
80. Domanska, M.K.; Dunning, R.A.; Dryden, K.A.; Zawada, K.E.; Yeager, M.; Kasson, P.M. Hemagglutinin spatial distribution shifts in response to cholesterol in the influenza viral envelope. *Biophys J.* **2015**, *109*, 1917–1924.
81. Chlanda, P.; Mekhedov, E.; Waters, H.; Schwartz, C.L.; Fischer, E.R.; Ryham, R.J.; Cohen, F.S.; Blank, P.S.; Zimmerberg, J. The hemifusion structure induced by influenza virus haemagglutinin is determined by physical properties of the target membranes. *Nat. Microbiol.* **2016**, *1*, 16050.
82. Mair, C.M.; Ludwig, K.; Herrmann, A.; Sieben, C. Receptor binding and pH stability – how influenza A virus hemagglutinin affects host-specific virus infection. *Biochim. Biophys. Acta* **2014**, *1838*, 1153–1168.
83. Russell, C.J. Acid-induced membrane fusion by the hemagglutinin protein and its role in influenza virus biology. *Cur.r Top. Microbiol. Immunol.* **2014**, *385*, 93–116.
84. Reed, M.L.; Bridges, O.A.; Seiler, P.; Kim, J.K.; Yen, H.L.; Salomon, R.; Govorkova, E.A.; Webster, R.G.; Russell, C.J. The pH of activation of the hemagglutinin protein regulates H5N1 influenza virus

pathogenicity and transmissibility in ducks. *J. Virol.* **2010**, *84*, 1527–1535.
85. Wang, W.; DeFeo, C.J.; Alvarado-Facundo, E.; Vassell, R.; Weiss, C.D. Intermonomer interactions in hemagglutinin subunits HA1 and HA2 affecting hemagglutinin stability and influenza virus infectivity. *J. Virol.* **2015**, *89*, 10602–10611.
86. Kemble, G.W.; Danieli, T.; and White, J.M. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. *Cell* **1994**, *76*, 383–391.
87. Kemble, G.W.; Bodian, D.L.; Rose, J.; Wilson, I.A.; White, J.M. Intermonomer disulfide bonds impair the fusion activity of influenza virus hemagglutinin. *J. Virol.* **1992**, *66*, 4940–4950.
88. Xu, S.; Zhou, J.; Liu, K.; Liu, Q.; Xue, C.; Li, X.; Zheng, J.; Luo, D.; Cao, Y. Mutations of two transmembrane cysteines of hemagglutinin (HA) from influenza A H3N2 virus affect HA thermal stability and fusion activity. *Virus Genes* **2013**, *47*, 20–26.
89. Zhou, J.; Xu, S.; Ma, J.; Lei, W.; Liu, K.; Liu, Q.; Ren, Y.; Xue, C.; Cao, Y. Recombinant influenza A H3N2 viruses with mutations of HA transmembrane cysteines exhibited altered virological characteristics. *Virus Genes* **2014**, *48*, 273–282.
90. Chlanda, P.; Mekhedov, E.; Waters, H.; Sodt, A.; Schwartz, C.; Nair, V.; Blank, P.S.; Zimmerberg, J. Palmitoylation Contributes to Membrane Curvature in Influenza A Virus Assembly and Hemagglutinin-Mediated Membrane Fusion. *J. Virol.* **2017**, *91*, e00947–17.
91. Xu, S.; Zhou, J.; Liu, Q.; Liu, K.; Xue, C.; Li, X.; Zheng, J.; Luo, D.; Cao, Y. Evidences for the existence of intermolecular disulfide-bonded oligomers in the H3 hemagglutinins expressed in insect cells. *Virus Genes* **2014**, *48*, 304–311.
92. Veit, M. Palmitoylation of virus proteins. *Biol. Cell* **2012**, *104*, 493–515.
93. Khurana, S.; Verma, S.; Verma, N.; Crevar, C.J.; Carter, D.M.; Manischewitz, J.; King, L.R.; Ross, T.M.; Golding, H. Bacterial HA1 vaccine against pandemic H5N1 influenza virus: Evidence of oligomerization, hemagglutination, and cross protective immunity in ferrets. *J. Virol.* **2011**, *85*, 1246–1256.
94. Chiu, C.; Ellebedy, A.H.; Wrammert, J.; Ahmed, R. B cell responses to influenza infection and vaccination. *Curr. Top. Microbiol. Immunol.* **2015**, *386*, 381–398.
95. Sano, K.; Ainai, A.; Suzuki, T.; Hasegawa, H. The road to a more effective influenza vaccine: Up to date studies and future prospects. *Vaccine* **2017**, *35*, 5388–5395.
96. Cotter, C.R.; Jin, H.; Chen, Z. A single amino acid in the stalk region of the H1N1pdm influenza virus HA protein affects viral fusion, stability and infectivity. *PLoS Patho*g. **2014**, *10*, e1003831.
97. Wei, C.J.; Xu, L.; Kong, W.P.; Shi, W.; Canis, K.; Stevens, J.; Yang, ZY.; Dell, A.; Haslam, S.M.; Wilson, I.A.; et al. Comparative efficacy of neutralizing antibodies elicited by recombinant hemagglutinin proteins from avian H5N1 influenza virus. *J. Virol.* **2008**, *82*, 6200–6208.
98. Weldon, W.C.; Wang, B.Z.; Martin, M.P.; Koutsonanos, D.G.; Skountzou, I.; Compans, R.W. Enhanced immunogenicity of stabilized trimeric soluble influenza hemagglutinin. *PloS ONE* **2010**, *5*, e12466.
99. Kanekiyo, M.; Wei, C.J.; Yassine, H.M.; McTamney, P.M.; Boyington, J.C.; Whittle, J.R.; Rao, S.S.; Kong, W.P.; Wang, L.; Nabel, G.J. Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies. *Nature* **2013**, *499*, 102–106.
100. Wang, W.; Song, H.S.; Keller, P.W.; Alvarado-Facundo, E.; Vassell, R.; Weiss, C.D. Conformational stability of the hemagglutinin of H5N1 influenza A viruses influences susceptibility to broadly neutralizing stem antibodies. *J. Virol.* **2018**, *92*, e00247–18.
101. Air, G.M. Influenza virus antigenicity and broadly neutralizing epitopes. *Curr. Opin. Virol.* **2015**, *11*, 113–121.
102. Joyce, M.G.; Wheatley, A.K.; Thomas, P.V.; Chuang, G.Y.; Soto, C.; Bailer, R.T.; Druz, A.; Georgiev, I.S.; Gillespie, R.A.; Kanekiyo, M.; et al. Vaccine-induced antibodies that neutralize group 1 and group 2 influenza A viruses. *Cell* **2016**, *166*, 609–623.
103. Andrews, S.F.; Graham, B.S.; Mascola, J.R.; McDermott, A.B. Is it possible to develop a "universal" influenza virus vaccine? Immunogenetic considerations underlying B-cell biology in the development of a pan-subtype influenza A vaccine targeting the hemagglutinin stem. *Cold Spring Harb. Perspect. Biol.* **2017**, *10*, a029413.
104. Kashyap, A.K.; Steel, J.; Oner, A.F.; Dillon, M.A.; Swale, R.E.; Wall, K.M.; Perry, K.J.; Faynboym, A.; Ilhan, M.; Horowitz, M.; et al. Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 5986–5991.
105. Corti, D.; Cameroni, E.; Guarino, B.; Kallewaard, N.L.; Zhu, Q.; Lanzavecchia, A. Tackling influenza with broadly neutralizing antibodies. *Curr. Opin. Virol.* **2017**, *24*, 60–69.
106. Wu, N.C.; Wilson, I.A. Structural insights into the design of novel anti-influenza therapies. *Nat. Struct. Mol. Biol.* **2018**, *25*, 115–121.
107. Chen, Y.Q.; Wohlbold, T.J.; Zheng, N.Y.; Huang, M.; Huang, Y.; Neu, K.E.; Lee, J.; Wan, H.; Rojas, K.T.; Kirkpatrick, E.; et al. Influenza Infection in Humans Induces Broadly Cross-Reactive and Protective

Neuraminidase-Reactive Antibodies. *Cell* **2018**, *173*, 417–429.
108. Ekiert, D.C.; Kashyap, A.K.; Steel, J.; Rubrum, A.; Bhabha, G.; Khayat, R.; Lee, J.H.; Dillon, M.A.; O'Neil, R.E.; Faynboym, A.M.; et al. Cross-neutralization of influenza A viruses mediated by a single antibody loop. *Nature* **2012**, *489*, 526–532.
109. Lee, P.S.; Yoshida, R.; Ekiert, D.C.; Sakai, N.; Suzuki, Y.; Takada, A.; Wilson, I.A. Heterosubtypic antibody recognition of the influenza virus hemagglutinin receptor binding site enhanced by avidity. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 17040–17045.
110. Schmidt, A.G.; Xu, H.; Khan, A.R.; O'Donnell, T.; Khurana, S.; King, L.R.; Manischewitz, J.; Golding, H.; Suphaphiphat, P.; Carfi, A.; et al. Preconfiguration of the antigen-binding site during affinity maturation of a broadly neutralizing influenza virus antibody. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 264–269.
111. McCarthy, K.R.; Watanabe, A.; Kuraoka, M.; Do, K.T.; McGee, C.E.; Sempowski, G.D.; Kepler, T.B.; Schmidt, A.G.; Kelsoe, G.; Harrison, S.C. Memory B cells that cross-react with group 1 and group 2 influenza A viruses are abundant in adult human repertoires. *Immunity* **2018**, *48*, 174–184.
112. Wu, N.C.; Grande, G.; Turner, H.L.; Ward, A.B.; Xie, J.; Lerner, R.A.; Wilson, I.A. In vitro evolution of an influenza broadly neutralizing antibody is modulated by hemagglutinin receptor specificity. *Nat. Commun.* **2017**, *8*, 15371.
113. Ekiert, D.C.; Bhabha, G.; Elsliger, M.A.; Friesen, R.H.; Jongeneelen, M.; Throsby, M.; Goudsmit, J.; Wilson, I.A. Antibody recognition of a highly conserved influenza virus epitope. *Science* **2009**, *324*, 246–251.
114. Corti, D.; Voss, J.; Gamblin, S.J.; Codoni, G.; Macagno, A.; Jarrossay, D.; Vachieri, S.G.; Pinna, D.; Minola, A.; Vanzetta, F.; et al. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science* **2011**, *333*, 850–856.
115. Dreyfus, C.; Laursen, N.S.; Kwaks, T.; Zuijdgeest, D.; Khayat, R.; Ekiert, D.C.; Lee, J.H.; Metlagel, Z.; Bujny, M.V.; Jongeneelen, M.; et al. Highly conserved protective epitopes on influenza B viruses. *Science* **2012**, *337*, 1343–1348.
116. Laursen, N.S.; Friesen, R.H.E.; Zhu, X.; Jongeneelen, M.; Blokland, S.; Vermond, J.; van Eijgen, A.; Tang, C.; van Diepen, H.; Obmolova, G.; et al. Universal protection against influenza infection by a multidomain antibody to influenza hemagglutinin. *Science* **2018**, *362*, 598–602.
117. Balazs, A.B.; Bloom, J.D.; Hong, C.M.; Rao, D.S.; Baltimore, D. Broad protection against influenza infection by vectored immunoprophylaxis in mice. *Nat. Biotechnol.* **2013**, *31*, 647–652.
118. Yoshida, R.; Igarashi, M.; Ozaki, H.; Kishida, N.; Tomabechi, D.; Kida, H.; Ito, K.; Takada, A. Cross-protective potential of a novel monoclonal antibody directed against antigenic site B of the hemagglutinin of influenza A viruses. *PLoS Pathog.* **2009**, *5*, e1000350.
119. Nakamura, G.; Chai, N.; Park, S.; Chiang, N.; Lin, Z.; Chiu, H.; Fong, R.; Yan, D.; Kim, J.; Zhang. J.; et al. An in vivo human-plasmablast enrichment technique allows rapid identification of therapeutic influenza A antibodies. *Cell Host Microbe* **2013**, *14*, 93–103.
120. Whittle, J.R.; Zhang, R.; Khurana, S.; King, L.R.; Manischewitz, J.; Golding, H.; Dormitzer, P.R.; Haynes, B.F.; Walter, E.B.; Moody, M.A.; et al. Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 14216–14221.
121. Bangaru, S.; Zhang, H.; Gilchuk, I.M.; Voss, T.G.; Irving, R.P.; Gilchuk, P.; Matta, P.; Zhu, X.; Lang, S.; Nieusma, T.; et al. A multifunctional human monoclonal neutralizing antibody that targets a unique conserved epitope on influenza HA. *Nat. Commun.* **2018**, *9*, 2669.
122. Amanat, F.; Meade, P.; Strohmeier, S.; Krammer, F. Cross-reactive antibodies binding to H4 hemagglutinin protect against a lethal H4N6 influenza virus challenge in the mouse model. *Emerg. Microbes Infect.* **2019**, *8*, 155–168.
123. Shen, C.; Chen, J.; Li, R.; Zhang, M.; Wang, G.; Stegalkina, S.; Zhang, L.; Chen, J.; Cao, J.; Bi, X.; et al. A multimechanistic antibody targeting the receptor binding site potently cross-protects against influenza B viruses. *Sci. Transl. Med.* **2017**, *9*, eaam5752.
124. Shen, C.; Zhang, M.; Chen, Y.; Zhang, L.; Wang, G.; Chen, J.; Chen, S.; Li, Z.; Wei, F.; Chen, J.; et al. An IgM antibody targeting the receptor binding site of influenza B blocks viral infection with great breadth and potency. *Theranostics* **2019**, *9*, 210–231.
125. Liu, Y.; Tan, H.X.; Koutsakos, M.; Jegaskanda, S.; Esterbauer, R.; Tilmanis, D.; Aban, M.; Kedzierska, K.; Hurt, A.C.; Kent. S.J.; et al. Cross-lineage protection by human antibodies binding the influenza B hemagglutinin. *Nat. Commun.* **2019**, *10*, 324.
126. Zuo, T.; Sun, J.; Wang, G.; Jiang, L.; Zuo, Y.; Li, D.; Shi, X.; Liu, X.; Fan, S.; Ren, H.; et al. Comprehensive analysis of antibody recognition in convalescent humans from highly pathogenic avian influenza H5N1 infection. *Nat. Commun.* **2015**, *6*, 8855.
127. Zuo, Y.; Wang, P.; Sun, J.; Guo, S.; Wang, G.; Zuo, T.; Fan, S.; Zhou, P.; Liang, M.; Shi, X.; et al. Complementary recognition of the receptor-binding site of highly pathogenic H5N1 influenza viruses by two human neutralizing antibodies. *J. Biol. Chem.* **2018**, *293*, 16503–16517.

128. Wang, P.; Zuo, Y.; Sun, J.; Zuo, T.; Zhang, S.; Guo, S.; Shi, X.; Liang, M.; Zhou, P.; Zhang, L.; et al. Structural and functional definition of a vulnerable site on the hemagglutinin of highly pathogenic avian influenza A virus H5N1. *J. Biol Chem.* **2019**, *294*, 4290–4303.
129. Gronsang, D.; Bui, A.N.; Trinh, D.Q.; Bui, V.N.; Nguyen, K.V.; Can, M.X.; Omatsu, T.; Mizutani, T.; Nagai, M.; Katayama, Y.; et al. Characterization of cross-clade monoclonal antibodies against H5N1 highly pathogenic avian influenza virus and their application to the antigenic analysis of diverse H5 subtype viruses. *Arch. Virol.* **2017**, *162*, 2257–2269.
130. Fouchier, R.A.; Munster, V.; Wallensten, A.; Bestebroer, T.M.; Herfst, S.; Smith, D.; Rimmelzwaan, G.F.; Olsen, B.; Osterhaus, A.D. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.* **2005**, *79*, 2814–2822.
131. Raymond, D.D.; Bajic, G.; Ferdman, J.; Suphaphiphat, P.; Settembre, E.C.; Moody, M.A.; Schmidt, A.G.; Harrison, S.C. Conserved epitope on influenza-virus hemagglutinin head defined by a vaccine-induced antibody. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 168–173.
132. Throsby, M.; van den Brink, E.; Jongeneelen, M; Poon, L.L.M.; Alard, P.; Cornelissen, L.; Bakker, A.; Cox, F.; van Deventer, E.; Guan, Y.; et al. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. *PLoS ONE* **2008**, *3*, e3942.
133. Lang, S.; Xie, J.; Zhu, X.; Wu, N.C.; Lerner, R.A.; Wilson, I.A. Antibody 27F3 broadly targets influenza A group 1 and group 2 hemagglutinins through a further variation in VH1–69 antibody orientation on the HA stem. *Cell Rep.* **2017**, *20*, 2935–2943.
134. Jacobsen, H.; Rajendran, M.; Choi, A.; Sjursen, H.; Brokstad, K.A.; Cox, R.J.; Palese, P.; Krammer, F.; Nachbagauer, R. Influenza virus hemagglutinin stalk-specific antibodies in human serum are a surrogate marker for in vivo protection in a serum transfer mouse challenge model. *MBio* **2017**, *8*, e01463–17.
135. Andrews, S.F.; Joyce, M.G.; Chambers, M.J.; Gillespie, R.A.; Kanekiyo, M.; Leung, K.; Yang, E.S.; Tsybovsky, Y.; Wheatley, A.K.; Crank, M.C.; et al. Preferential induction of cross-group influenza A hemagglutinin stem-specific memory B cells after H7N9 immunization in humans. *Sci. Immunol.* **2017**, *2*, eaan2676.
136. Bommakanti, G.; Lu, X.; Citron, M.P.; Najar, T.A.; Heidecker, G.J.; ter Meulen, J.; Varadarajan, R.; Liang, X. Design of Escherichia coli-expressed stalk domain immunogens of H1N1 hemagglutinin that protect mice from lethal challenge. *J. Virol.* **2012**, *86*, 13434–13444.
137. Krammer, F. and Palese, P. Influenza virus hemagglutinin stalk-based antibodies and vaccines. *Curr. Opin. Virol.* **2013**, *3*, 521–530.
138. Krammer, F.; Hai, R.; Yondola, M.; Tan, G.S.; Leyva-Grado, V.H.; Ryder, A.B.; Miller, M.S.; Rose, J.K.; Palese, P.; García-Sastre, A.; et al. Assessment of influenza virus hemagglutinin stalk-based immunity in ferrets. *J. Virol.* **2014**, *88*, 3432–3442.
139. Friesen, R.H.; Lee, P.S.; Stoop, E.J.; Hoffman, R.M.; Ekiert, D.C.; Bhabha, G.; Yu, W.; Juraszek, J.; Koudstaal, W.; Jongeneelen, M.; et al. A common solution to group 2 influenza virus neutralization. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 445–450.
140. Mallajosyula, V.V.; Citron, M.; Ferrara, F.; Temperton, N.J.; Liang, X.; Flynn, J.A.; Varadarajan, R. Hemagglutinin sequence conservation guided stem immunogen design from influenza A H3 subtype. *Front. Immunol.* **2015**, *6*, 329.
141. Kallewaard, N.L.; Corti, D.; Collins, P.J.; Neu, U.; McAuliffe, J.M.; Benjamin, E.; Wachter-Rosati, L.; Palmer-Hill, F.J.; Yuan, A.Q.; Walker, P.A.; et al. Structure and function analysis of an antibody recognizing all influenza A subtypes. *Cell* **2016**, *166*, 596–608.
142. Whitehead, T.A.; Chevalier, A.; Song, Y.; Dreyfus, C.; Fleishman, S.J.; De Mattos, C.; Myers, C.A.; Kamisetty, H.; Blair, P.; Wilson, I.A.; et al. Optimization of affinity, specificity and function of designed influenza inhibitors using deep sequencing. *Nat. Biotechnol.* **2012**, *30*, 543–548.
143. Fleishman, S.J.; Whitehead, T.A.; Ekiert, D.C.; Dreyfus, C.; Corn, J.E.; Strauch, E.M.; Wilson, I.A.; Baker, D. Computational design of proteins targeting the conserved stem region of influenza hemagglutinin. *Science* **2011**, *332*, 816–821.
144. DiLillo, D.J.; Tan, G.S.; Palese, P.; Ravetch, J.V. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcgammaR interactions for protection against influenza virus in vivo. *Nat. Med.* **2014**, *20*, 143–151.
145. Sui, J.; Hwang, W.C.; Perez, S.; Wei, G.; Aird, D.; Chen, L.M.; Santelli, E.; Stec, B.; Cadwell, G.; Ali, M.; et al. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat. Struct. Mol. Biol.* **2009**, *16*, 265–273.
146. Ekiert, D.C.; Friesen, R.H.; Bhabha, G.; Kwaks, T.; Jongeneelen, M.; Yu, W.; Ophorst, C.; Cox, F.; Korse, H.J.; Brandenburg, B.; et al. A highly conserved neutralizing epitope on group 2 influenza A viruses. *Science* **2011**, *333*, 843–850.
147. Tan, G.S.; Krammer, F.; Eggink, D.; Kongchanagul, A.; Moran, T.M.; Palese, P. A pan-H1 anti-hemagglutinin monoclonal antibody with potent broad-spectrum efficacy in vivo. *J. Virol.* **2012**, *86*, 6179–6188.
148. Ni, Y.; Guo, J.; Turner, D.; Tizard, I. Development of a novel dual-domain nanopartical antigen construct

for universal influenza vaccine. *Vaccine* **2017**, *35*, 7026–7032.
149. Corbett, K.S.; Moin, S.M.; Yassine, H.M.; Cagigi, A.; Kanekiyo, M.; Boyoglu-Barnum, S.; Myers, S.I.; Tsybovsky, Y.; Wheatley, A.K.; Schramm, C.A.; et al. Design of nanoparticulate group 2 influenza virus hemagglutinin stem antigens that activate unmutated ancestor B cell receptors of broadly neutralizing antibody lineages. *MBio* **2019**, *10*, e02810–18.
150. Li, Z.; Wan, Z.; Li, T.; Xie, Q.; Sun, H.; Chen, H.; Liang, G.; Shao, H.; Qin, A.; Ye, J. A novel linear epitope crossing group 1 and group 2 influenza A viruses located in the helix A of HA2 derived from H7N9. *Vet. Microbiol.* **2019**, *228*, 39–44.
151. Chiu, C.; Wrammert, J.; Li, G.M.; McCausland, M.; Wilson, P.C.; Ahmed, R. Cross-reactive humoral responses to influenza and their implications for a universal vaccine. *Ann. N. Y. Acad. Sci.* **2013**, *1283*, 13–21.
152. Sridhar, S.; Brokstad, K.A.; Cox, R.J. Influenza vaccination strategies: Comparing inactivated and live attenuated influenza vaccine. *Vaccine* **2015**, *3*, 373–389.
153. Ebrahimie, E.; Nurollah, Z.; Ebrahimi, M.; Hemmatzadeh, F.; Ignjatovic, J. Unique ability of pandemic influenza to downregulate the genes involved in neuronal disorders. *Mol. Biol. Rep.* **2015**, *42*, 1377–1390.
154. Tarigan, S.; Wibowo, M.H.; Indriani, R.; Sumarningsih, S.; Artanto, S.; Idris, S.; Durr, P.A.; Asmara, W.; Ebrahimie, E.; Stevenson, M.A.; et al. Field effectiveness of highly pathogenic avian influenza H5N1 vaccination in commercial layers in Indonesia. *PLoS ONE* **2018**, *13*, e0190947.
155. Quan, L.; Ji, C.; Ding, X.; Peng, Y.; Liu, M.; Sun, J.; Jiang, T.; Wu, A. Cluster-transition determining sites underlying the antigenic evolution of seasonal influenza viruses. *Mol. Biol. Evol.* **2019**, doi:10.1093/molbev/msz050.
156. Subramanian, R.; Graham, A.L.; Grenfell, B.T.; Arinaminpathy, N. Universal or specific? A modeling-based comparison of broad-spectrum influenza vaccines against conventional, strain-matched vaccines. *PLoS Comput. Biol.* **2016**, *12*, e1005204.
157. Maamary, J.; Wang, T.T.; Tan, G.S.; Palese, P.; Ravetch, J.V. Increasing the breadth and potency of response to the seasonal influenza virus vaccine by immune complex immunization. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 10172–10177.
158. Abente, E.J.; Rajao, D.S.; Gauger, P.C.; Vincent, A.L. Alphavirus-vectored hemagglutinin subunit vaccine provides partial protection against heterologous challenge in pigs. *Vaccine* **2019**, *37*, 1533–1539.
159. Sisteré-Oró, M.; Vergara-Alert, J.; Stratmann, T.; López-Serrano, S.; Pina-Pedrero, S.; Córdoba, L.; Pérez-Maillo, M.; Pleguezuelos, P.; Vidal, E.; Veljkovic, V.; et al. Conserved HA-peptide NG34 formulated in pCMV-CTLA4-Ig reduces viral shedding in pigs after a heterosubtypic influenza virus SwH3N2 challenge. *PLoS ONE* **2019**, *14*, e0212431.
160. Ndifon, W.; Wingreen, N.S.; Levin, SA. Differential neutralization efficiency of hemagglutinin epitopes, antibody interference, and the design of influenza vaccines. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 8701–8706.
161. Nara, P.L.; Tobin, G.J.; Chaudhuri, A.R.; Trujillo, J.D.; Lin, G.; Cho, M.W.; Levin, S.A.; Ndifon, W.; Wingreen, N.S. How can vaccines against influenza and other viral diseases be made more effective? *PLoS Biol.* **2010**, *8*, 1–4.
162. Petrie, J.G.; Parkhouse, K.; Ohmit, S.E.; Malosh, R.E.; Monto, A.S.; Hensley, S.E. Antibodies Against the Current Influenza A(H1N1) Vaccine Strain Do Not Protect Some Individuals From Infection With Contemporary Circulating Influenza A(H1N1) Virus Strains. *J. Infect. Dis.* **2016**, *214*, 1947–1951.
163. Yu, X.; Tsibane, T.; McGraw, P.A.; House, F.S.; Keefer, C.J.; Hicar, M.D.; Tumpey, T.M.; Pappas, C.; Perrone, L.A.; Martinez, O.; et al. Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. *Nature* **2008**, *455*, 532–536.
164. Zimmerman, R.K.; Nowalk, M.P.; Chung, J.; Jackson, M.L.; Jackson, L.A.; Petrie, J.G.; Monto, A.S.; McLean, H.Q.; Belongia, E.A.; Gaglani, M.; et al. 2014–2015 influenza vaccine effectiveness in the united states by vaccine type. *Clin. Infect. Dis.* **2016**, *63*, 1564–1573.
165. Zost, S.J.; Parkhouse, K.; Gumina, M.E.; Kim, K.; Diaz, Perez, S.; Wilson, P.C.; Treanor, J.J.; Sant, A.J.; Cobey, S.; Hensley, S.E. Contemporary H3N2 influenza viruses have a glycosylation site that alters binding of antibodies elicited by egg-adapted vaccine strains. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 12578–12583.
166. Kirkpatrick, E.; Qiu, X.; Wilson, P.C.; Bahl, J.; Krammer, F. The influenza virus hemagglutinin head evolves faster than the stalk domain. *Sci. Rep.* **2018**, *8*, 10432.
167. Klausberger, M.; Tscheliessnig, R.; Neff, S.; Nachbagauer, R.; Wohlbold, T.J.; Wilde, M.; Palmberger, D.; Krammer, F.; Jungbauer, A.; Grabherr, R. Globular head-displayed conserved influenza H1 hemagglutinin stalk epitopes confer protection against heterologous H1N1 virus. *PLoS ONE* **2016**, *11*, e0153579.
168. Ni, Y.; Guo, J.; Turner, D.; Tizard, I. An improved inactivated influenza vaccine with enhanced cross protection. *Front. Immunol.* **2018**, *9*, 1815.
169. Wei, C.J.; Boyington, J.C.; McTamney, P.M.; Kong, W.P.; Pearce, M.B.; Xu, L.; Andersen, H.; Rao, S.; Tumpey, T.M.; Yang, Z.Y.; et al. Induction of broadly neutralizing H1N1 influenza antibodies by vaccination. *Science*

2010, *329*, 1060–1064.
170. Babu, T.M.; Levine, M.; Fitzgerald, T.; Luke, C.; Sangster, M.Y.; Jin, H.; Topham, D.; Katz, J.; Treanor, J.; Subbarao, K. Live attenuated H7N7 influenza vaccine primes for a vigorous antibody response to inactivated H7N7 influenza vaccine. *Vaccine* **2014**, *32*, 6798–6804.
171. Wang, S.C.; Liao, H.Y.; Zhang, J.Y.; Cheng, T.R.; Wong, C.H. Development of a universal influenza vaccine using hemagglutinin stem protein produced from *Pichia pastoris*. *Virology* **2019**, *526*, 125–137.
172. Steel, J.; Lowen, A.C.; Wang, T.T.; Yondola, M.; Gao, Q.; Haye, K.; García-Sastre, A.; Palese, P. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. *MBio* **2010**, *1*, e00018–10.
173. Valkenburg, S.A.; Mallajosyula, V.V.; Li, O.T.; Chin, A.W.; Carnell, G.; Temperton, N.; Varadarajan, R.; Poon, L.L. Stalking influenza by vaccination with pre-fusion headless HA mini-stem. *Sci. Rep.* **2016**, *6*, 22666.
174. Krammer, F.; Pica, N.; Hai, R.; Margine, I.; Palese, P. Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies. *J. Virol.* **2013**, *87*, 6542–6550.
175. Ermler, M.E.; Kirkpatrick, E.; Sun, W.; Hai, R.; Amanat, F.; Chromikova, V.; Palese, P.; Krammer, F. Chimeric hemagglutinin constructs induce broad protection against influenza B virus challenge in the mouse model. *J. Virol.* **2017**, *91*, e00286–17.
176. Nachbagauer, R.; Kinzler, D.; Choi, A.; Hirsh, A.; Beaulieu, E.; Lecrenier, N.; Innis, B.L.; Palese, P.; Mallett, C.P.; Krammer, F. A chimeric haemagglutinin-based influenza split virion vaccine adjuvanted with AS03 induces protective stalk-reactive antibodies in mice. *NPJ Vaccines* **2016**, *1*, pii:16015.
177. Nachbagauer, R.; Liu, W.C.; Choi, T.J.; Wohlbold, T.J.; Atlas, T.; Rajendran, M.; Solórzano, A.; Berlanda-Scorza, F.; García-Sastre, A.; Palese, P.; et al. A universal influenza virus vaccine candidate confers protection against pandemic H1N1 infection in preclinical ferret studies. *NPJ Vaccines* **2017**, *2*, 1–13.
178. Mallajosyula, V.V.; Citron, M.; Ferrara, F.; Lu, X.; Callahan, C.; Heidecker, G.J.; Sarma, S.P.; Flynn, J.A.; Temperton, N.J.; Liang, X.; et al. Influenza hemagglutinin stem-fragment immunogen elicits broadly neutralizing antibodies and confers heterologous protection. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, E2514–E2523.
179. Yassine, H.M.; McTamney, P.M.; Boyington, J.C.; Ruckwardt, T.J.; Crank, M.C.; Smatti, M.K.; Ledgerwood, J.E.; Graham, B.S. Use of Hemagglutinin Stem Probes Demonstrate Prevalence of Broadly Reactive Group 1 Influenza Antibodies in Human Sera. *Sci. Rep.* **2018**, *8*, 8628.
180. Impagliazzo, A.; Milder, F.; Kuipers, H.; Wagner, M.V.; Zhu, X.; Hoffman, R.M.; van Meersbergen, R.; Huizingh, J.; Wanningen, P.; Verspuij, J.; et al. A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. *Science* **2015**, *349*, 1301–1306.
181. Wohlbold, T.J.; Nachbagauer, R.; Margine, I.; Tan, G.S.; Hirsh, A.; Krammer, F. Vaccination with soluble headless hemagglutinin protects mice from challenge with divergent influenza viruses. *Vaccine* **2015**, *33*, 3314–3321.
182. Yassine, H.M.; Boyington, J.C.; McTamney, P.M.; Wei, C.J.; Kanekiyo, M.; Kong, W.P.; Gallagher, J.R.; Wang, L.; Zhang, Y.; Joyce, M.G.; et al. Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. *Nat. Med.* **2015**, *21*, 1065–1070.
183. Van der Lubbe, J.E.M.; Verspuij, J.W.A.; Huizingh, J.; Schmit-Tillemans, S.P.R.; Tolboom, J.T.B.M.; Dekking, L.E.H.A.; Kwaks, T.; Brandenburg, B.; Meijberg, W.; Zahn, R.C.; et al. Mini-HA is superior to full length hemagglutinin immunization in inducing stem-specific antibodies and protection against group 1 influenza virus challenges in mice. *Front. Immunol.* **2018**, *9*, 2350.
184. Margine, I.; Krammer, F.; Hai, R.; Heaton, N.S.; Tan, G.S.; Andrews, S.A.; Runstadler, J.A.; Wilson, P.C.; Albrecht, R.A.; García-Sastre, A.; et al. Hemagglutinin stalk-based universal vaccine constructs protect against group 2 influenza A viruses. *J. Virol.* **2013**, *87*, 10435–10446.
185. Nachbagauer, R.; Miller, M.S.; Hai, R.; Ryder, A.B.; Rose, J.K.; Palese, P.; García-Sastre, A.; Krammer, F.; Albrecht, R.A. Hemagglutinin Stalk Immunity Reduces Influenza Virus Replication and Transmission in Ferrets. *J. Virol.* **2015**, *90*, 3268–3273.
186. Isakova-Sivak, I.; Korenkov, D.; Smolonogina, T. Broadly protective anti-hemagglutinin stalk antibodies induced by live attenuated influenza vaccine expressing chimeric hemagglutinin. *Virology* **2018**, *518*, 313–323.
187. Andrews, S.F.; Huang, Y.; Kaur, K.; Popova, L.I.; Ho, I.Y.; Pauli, N.T.; Henry, Dunand, C.J.; Taylor, W.M.; Lim S1, Huang, M.; et al. Immune history profoundly affects broadly protective B cell responses to influenza. *Sci. Transl. Med.* **2015**, *7*, 316ra192.
188. Kordyukova, L. Structural and functional specificity of influenza viruses haemagglutinin and paramyxovirus fusion protein anchoring peptides. *Virus Res.* **2017**, *227*, 183–199.
189. Takeda, M.; Leser, G.P.; Russell, C.J.; Lamb, R.A. Influenza virus hemagglutinin concentrates in lipid raft microdomains for efficient viral fusion. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 14610–14617.
190. Tall, R.D.; Alonso, M.A.; Roth, M.G. Features of influenza HA required for apical sorting differ from those required for association with DRMs or MAL. *Traffic* **2003**, *4*, 838–849.

191. Rossman, J.S.; Lamb, R.A. Influenza virus assembly and budding. *Virology* **2011**, *411*, 229–236.
192. Zhang, J.; Pekosz, A.; Lamb, R.A. Influenza virus assembly and lipid raft microdomains: A role for the cytoplasmic tails of the spike glycoproteins. *J. Virol.* **2000**, *74*, 4634–4644.
193. Chen, B.J.; Takeda, M.; Lamb, R.A. Influenza virus hemagglutinin (H3 subtype) requires palmitoylation of its cytoplasmic tail for assembly: M1 proteins of two subtypes differ in their ability to support assembly. *J. Virol.* **2005**, *79*, 13673–13684.
194. Yang, H.; Carney, P.J.; Donis, R.O.; Stevens, J. Structure and receptor complexes of the hemagglutnin from a highly pathogenic H7N7 influenza virus. *J. Virol.* **2012**, *86*, 8645–8652.
195. Ting-Hui-Lin, Chia, M.Y.; Lin, C.Y.; Yeh, Y.Q.; Jeng, U.S.; Wu, W.G.; Lee, M.S. Improving immunogenicity of influenza virus H7N9 recombinant hemagglutinin for vaccine development. *Vaccine* **2019**, *37*, 1897–1903.
196. Liu, Q.L.; Xue, C.Y.; Zheng, J.; Liu, K.; Wang, Y.; Wei, Y.; Liu, G.D.; Cao, Y. Influenza bivalent vaccine comprising recombinant H3 hemagglutinin (HA) and H1 HA containing replaced H3 hemagglutinin transmembrane domain exhibited improved heterosubtypic protection immunity in mice. *Vaccine* **2015**, *33*, 4035–4040.
197. Wang, Y.; Zhang, Y.; Wu, J.; Lin, Y.; Wu, Z.; Wei, Y.; Wei, X.; Qin, J.; Xue, C.; Liu, G.D.; et al. Recombinant influenza H7 hemagglutinin containing CFLLC minidomain in the transmembrane domain showed enhanced cross-protection in mice. *Virus Res.* **2017**, *242*, 16–23.
198. Wang, Y.; Wu, J.; Xue, C.; Wu, Z.; Lin, Y.; Wei, Y.; Wei, X.; Qin, J.; Zhang, Y.; Wen, Z.; et al. A recombinant H7N9 influenza vaccine with the H7 hemagglutinin transmembrane domain replaced by the H3 domain induces increased cross-reactive antibodies and improved interclade protection in mice. *Antiviral Res.* **2017**, *143*, 97–105.
199. Zhang, Y.; Wei, Y.; Liu, K.; Huang, M.; Li, R.; Wang, Y.; Liu, Q.; Zheng, J.; Xue, C.; Cao, Y. Recombinant influenza H9N2 virus with a substitution of H3 hemagglutinin transmembrane domain showed enhanced immunogenicity in mice and chicken. *Sci. Rep.* **2017**, *7*, 17923.
200. Qin, J.; Zhang, Y.; Shen, X; Gong, L.; Peng, O.; Liu, Y.; Xue, C.; Cao, Y. H7 virus-like particles assembled by hemagglutinin containing H3N2 transmembrane domain and M1 induce broad homologous and heterologous protection in mice. *Vaccine* **2018**, *36*, 5030–5036.
201. Qin, J.; Zhang, Y.; Shen, X.; Gong, L.; Xue, C.; Cao, Y. Biological characteristics and immunological properties in Muscovy ducks of H5N6 virus-like particles composed of HA-TM/HA-TM_{H3} and M1. *Avian Pathol.* **2019**, *48*, 35–44.
202. Wang, J.; Wu, Y.; Ma, C.; Fiorin, G.; Wang, J.; Pinto, L.H.; Lamb, R.A.; Klein, M.L.; Degrado, W.F. Structure and inhibition of the drug-resistant S31N mutant of the M2 ion channel of influenza A virus. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 1315–1320.
203. Das, K.; Aramini, J.M.; Ma, L.C.; Krug, R.M.; Arnold, E. Structures of influenza A proteins and insights into antiviral drug targets. *Nat. Struct. Mol. Biol.* **2010**, *17*, 530–538.
204. Palmer, R. Drugs: Lines of defence. *Nature* **2011**, *480*, S9–S10.
205. Sugrue, R.J.; Tan, B.H.; Yeo, D.S.Y.; Sutejo, R. Antiviral drugs for the control of pandemic influenza virus. *Ann. Acad. Med. Singap.* **2008**, *37*, 518–524.
206. Spanakis, N.; Pitiriga, V.; Gennimata, V.; Tsakris, A. A review of neuraminidase inhibitor susceptibility in influenza strains. *Expert Rev. Antiinfect. Ther.* **2014**, *12*, 1325–1336.
207. Skehel, J.J.; Wiley, D.C. Receptor Binding and Membrane Fusion in Virus Entry: The Influenza Hemagglutinin. *Annu. Rev. Biochem.* **2000**, *69*, 531–569.
208. Plotch, S.J.; O'Hara, B.; Morin, J.; Palant, O.; LaRocque, J.; Bloom, J.D.; Lang, S.A. Jr.; DiGrandi, M.J.; Bradley, M.; Nilakantan, R.; et al. Inhibition of influenza A virus replication by compounds interfering with the fusogenic function of the viral hemagglutinin. *J. Virol.* **1999**, *73*, 140–151.
209. Luo, G.; Colonno, R.; Krystal, M. Characterization of a hemagglutinin-specific inhibitor of influenza A virus. *Virology* **1996**, *226*, 66–76.
210. Staschke, K.A; Hatch, S.D.; Tang, J.C.; Hornback, W.J.; Munroe, J.E.; Colacino, J.M.; Muesing, M.A. Inhibition of influenza virus hemagglutinin mediated membrane fusion by a compound related to podocarpic acid. *Virology* **1998**, *248*, 264–274.
211. Zhu, L.; Li, Y.; Li, S.; Li, H.; Qiu, Z.; Lee, C.; Lu, H.; Lin, X.; Zhao, R.; Chen, L.; et al. Inhibition of influenza A virus (H1N1) fusion by benzenesulfonamide derivatives targeting viral hemagglutinin. *PLoS ONE* **2011**, *6*, e29120.
212. Lai, K.K.; Cheung, N.N.; Yang, F.; Dai, J.; Liu, L.; Chen, Z.; Sze, K.H.; Chen, H.; Yuen, K.Y.; Kao, R.Y. Identification of novel fusion inhibitors of influenza A virus by chemical genetics. *J. Virol.* **2015**, *90*, 2690–2701.
213. Bodian, D.L.; Yamasaki, R.B.; Buswell, R.L.; Stearns, J.F.; White, J.M.; Kuntz, I.D. Inhibition of the fusion-inducing conformational change of influenza hemagglutinin by benzoquinones and hydroquinones. *Biochemistry* **1993**, *32*, 2967–2978.

214. Hoffman, L.R.; Kuntz, I.D.; White, J.M. Structure-based identification of an inducer of the low-pH conformational change in the influenza virus hemagglutinin: Irreversible inhibition of infectivity. *J. Virol.* **1997**, *71*, 8808–8820.
215. Kingsley, C.N.; Antanasijevic, A.; Palka-Hamblin, H.; Durst, M.; Ramirez, B.; Lavie, A.; Caffrey, M. Probing the metastable state of influenza hemagglutinin. *J. Biol. Chem.* **2017**, *292*, 21590–21597.
216. Leneva, I.A.; Russell, R.J.; Boriskin, Y.S.; Hay, A.J. Characteristics of arbidol-resistant mutants of influenza virus: Implications for the mechanism of anti-influenza action of arbidol. *Antiviral Res.* **2009**, *81*, 132–140.
217. Kadam, R.U.; Wilson, I.A. Structural basis of influenza virus fusion inhibition by the antiviral drug arbidol. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 206–214.
218. Van Dongen, M.J.P.; Kadam, R.U.; Juraszek, J.; Lawson, E.; Brandenburg, B.; Schmitz, F.; Schepens, W.B.G.; Stoops, B.; van Diepen, H.A.; Jongeneelen, M.; et al. A small-molecule fusion inhibitor of influenza virus is orally active in mice. *Science* **2019**, *363*, eaar6221.
219. Król, E.; Rychłowska, M.; Szewczyk, B. Antivirals – current trends in fighting influenza. *Acta Biochim. Pol.* **2014**, *61*, 495–504.
220. Zavyalova, E.G.; Kopylov, A.M. Aptamers to hemagglutinin: A novel tool for influenza virus recognition and neutralization. *Curr. Pharm. Des.* **2015**, *22*, 4835–4853.
221. Masson, G.R.; Jenkins, M.L.; Burke, J.E. An overview of hydrogen deuterium exchange mass spectrometry (HDX-MS) in drug discovery. *Exp. Opin. Drug Discov.* **2017**, *12*, 981–994.
222. Puchades, C.; Kükrer; B.; Diefenbach, O; et al. Epitope mapping of diverse influenza Hemagglutinin drug candidates using HDX-MS. *Sci. Rep.* **2019**, *9*, 4735.
223. Soema, P.C.; van Riet, E.; Kersten, G.; Amorij, J.P. Development of cross-protective influenza A vaccines based on cellular responses. *Front. Immunol.* **2015**, *6*, 237.
224. Kanekiyo, M.; Joyce, M.G.; Gillespie, R.A.; Gallagher, J.R.; Andrews, S.F.; Yassine, H.M.; Wheatley, A.K.; Fisher, B.E.; Ambrozak, D.R.; Creanga, A.; et al. Mosaic nanoparticle display of diverse influenza virus hemagglutinins elicits broad B cell responses. *Nat. Immunol.* **2019**, *20*, 362–372.
225. Wang, S.H.; Smith, D.; Cao, Z.; Chen, J.; Acosta, H.; Chichester, J.A.; Yusibov, V.; Streatfield, S.J.; Fattom, A.; Baker, J.R. Jr. Recombinant H5 hemagglutinin adjuvanted with nanoemulsion protection ferrets against pathogenic avian influenza virus challenge. *Vaccine* **2019**, *37*, 1591–1600.
226. Kimoto, T.; Kim, H.; Sakai, S.; Takahashi, E.; Kido, H. Oral vaccination with influenza hemagglutinin combined with human pulmonary surfactant-mimicking synthetic adjuvant SF-10 induces efficient local and systemic immunity compared with nasal and subcutaneous vaccination and provides protective immunity in mice. *Vaccine* **2019**, *37*, 612–622.
227. Allen, J.D.; Owino, S.O.; Carter, D.M.; Crevar, C.J.; Reese, V.A.; Fox, C.B.; Coler, R.N.; Reed, S.G.; Baldwin, S.L.; Ross, T.M. Broadened immunity and protective responses with emulsion-adjuvanted H5 COBRA-VLP vaccines. *Vaccine* **2017**, *35*, 5209–5216.
228. Carter, D.M.; Darby, C.A.; Lefoley, B.C.; Crevar, C.J.; Alefantis, T.; Oomen, R.; Anderson, S.F.; Strugnell, T.; Cortés-Garcia, G.; Vogel, T.U.; et al. Design and characterization of a computationally optimized broadly reactive hemagglutinin vaccine for H1N1 influenza viruses. *J. Virol.* **2016**, *90*, 4720–4734.
229. Carter, D.M.; Darby, C.A.; Johnson, S.K.; Carlock, M.A.; Kirchenbaum, G.A.; Allen, J.D.; Vogel, T.U.; Delagrave, S.; DiNapoli, J.; Kleanthous, H.; et al. Elicitation of protective antibodies against a broad panel of H1N1 viruses in ferrets preimmune to historical H1N1 influenza viruses. *J. Virol.* **2017**, *91*, e01283-17.
230. Allen, J.D.; Ray, S.; Ross, T.M. Split inactivated COBRA vaccine elicits protective antibodies against H1N1 and H3N2 influenza viruses. *PLoS ONE* **2018**, *13*, e0204284.
231. Ross, T.M.; DiNapoli, J.; Giel-Moloney, M.; Bloom, C.E.; Bertran, K.; Balzli, C.; Strugnell, T.; Sá E.; Silva, M.; Mebatsion, T.; Bublot, M.; et al. A computationally designed H5 antigen shows immunological breadth of coverage and protects against drifting avian strains. *Vaccine* **2019**, *37*, 2369–2376.
232. Wong, S.S.; Webby, R.J. Traditional and new influenza vaccines. *Clin. Microbiol. Rev.* **2013**, *26*, 476–492.
233. Heinen, P.P.; Rijsewijk, F.A.; de Boer-Luijtze, E.A.; Bianchi, A.T. Vaccination of pigs with a DNA construct expressing an influenza virus M2-nucleoprotein fusion protein exacerbates disease after challenge with influenza A virus. *J. Gen. Virol* .**2002**, *83*, 1851–1859.