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Identification of Influenza Hemagglutinin Residues Critical for Membrane Fusion Activity

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By

Jessica Trost

B.S., University of Wisconsin-Madison, 2007

Advisor: David Steinhauer, PhD

An abstract of

A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

Graduate Division of Biological and Biomedical Science

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2020

Abstract

Identification of Influenza Hemagglutinin Residues Critical for Membrane Fusion Activity

By Jessica Trost

Influenza is a major public health risk causing annual seasonal epidemics and sporadic pandemics when a subtype transmits to naïve human population and circulates. Current vaccine strategies are limited, requiring annual immunization and evaluation of included strains. The hemagglutinin (HA), one of the major surface glycoproteins of influenza A virus, is the principle target for vaccination. HA functions in the viral life cycle both in the binding of the virus to host cells and in mediating fusion of the viral and endosomal membranes, via a low pH-induced conformational change. The structural rearrangements of the HA during this conformational change are critical to viral entry, and mutations in the protein can impact the pH at which fusion occurs and the overall stability of the HA structure. HA stability can play a role in viral pathogenesis, host range, and adaptation to new hosts. The HA is expressed as a homotrimeric spike on viral surfaces, with a membrane-distal globular head domain situated on a fibrous stalk, or stem domain. Current vaccines focus immunity on the HA head, which undergoes frequent mutations during “antigenic drift”. The HA stalk is more conserved and therefore an attractive target for universal vaccine strategies and antiviral drug design. The 16 antigenic HA subtypes of naturally circulating influenza A viruses are segregated into two groups, group-1 and group-2. For the group-2 HAs, previous studies identified specific residues within the HA stalk as potential “trigger” residues for membrane fusion-associated conformational changes. However, for group-1 HAs no such residues have been conclusively implicated. Based on comparative sequence analyses and structural considerations, a series of experiments were designed to gain insights into the initiation of conformational changes for group-1 HAs. Functional studies on mutant HAs of several subtypes revealed that a histidine at residue 111 within the HA2 subunit of group-1 HA types was required for acid-induced conformational changes and membrane fusion activity. As this histidine is completely conserved in natural isolates of group-1 HAs, it might serve as an “Achilles’ heel” residue to target in future vaccine and antiviral strategies.

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There are many people who have contributed to my success and believed in me. For that I hope this serves to show how much of their efforts and words allowed me to persevere and continue to climb obstacles and challenges. Thank you all for being

there for me along the way, you have been a part of this journey and I know it would not have been possible without you. As I conclude, let me leave you with the words that are so iconic to the Steinhauer laboratory: Cheers!

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Introduction

Background and History

Influenza viruses are a member of the *Orthomyxoviridae* family which includes negative-sense, single-stranded RNA viruses with segmented genomes. There are seven genera within the family of *Orthomyxoviridae;* four of these genera encompass the influenza A, B, C and D viruses (IAV, IBV, ICV, and IDV) based on the antigenic properties of their nucleoprotein (NP) and matrix (M) proteins [1, 2]. IAV, IBV, and ICV can all cause human disease, whereas IDV is primarily found in porcine and bovine species. In most years, IAV are the major cause of human influenza disease and are the viruses responsible for all human influenza pandemics of the past century.

IAV are classified using nomenclature based on the antigenic properties of the two major surface glycoproteins, Hemagglutinin (HA) and Neuraminidase (NA) [3, 4]. The HAs can be segregated into 16 different subtypes, all found in the natural reservoir of aquatic birds, H1-H16; while the NA has nine different subtypes, N1-N9. Evidence of H17 and H18 as well as N10 and N11 in bats has been shown through isolation of RNA [5]. Based on sequence and structural criteria, the HA subtypes are further divided into group-1, which includes H1, H2, H5, H6, H7, H8, H9, H11, H13, and H16 subtypes, and group-2, which includes H3, H4, H7, H10, H14, and H15 subtypes [6, 7]. Amino acid variation within each of the subtypes can range anywhere from 0-9%, while inter-subtype variation of amino acids can range from 20-74%. Notable residues, such as cysteines that participate in disulfide bonds, are conserved, suggesting evolution from a progenitor hemagglutinin gene [7]. Although amino acid sequence identity among subtypes and within the two groups can be as little as 30%, the overall structure and

functions of the hemagglutinin are similar among all the HA subtypes, as shown in Figure 1.

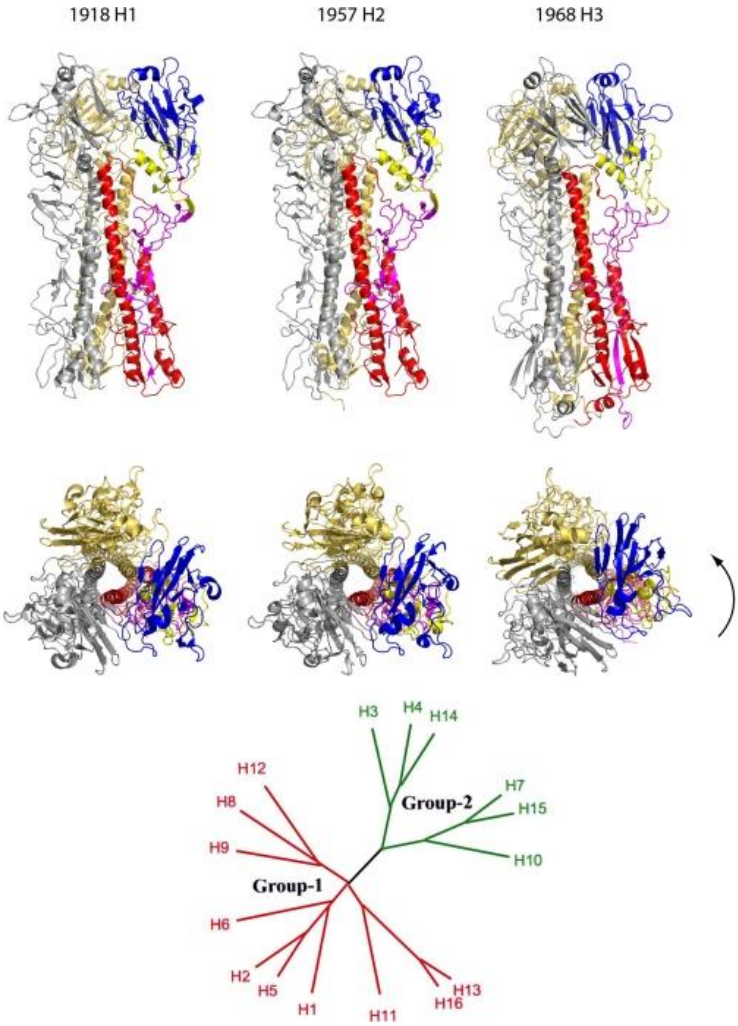

Figure 1. Phylogenetic comparisons of group-1 HA subtypes H1 and H2 and group-2 HA subtype H3. The top shows the crystal structure of the trimeric form of the HA proteins in ribbon format. The blue color corresponds to the receptor binding site of the HA, the yellow represents the esterase, and the red and magenta colors highlight fusion subdomains. The middle panel shows a top-down view of the HA trimer structure. The bottom panel shows the phylogenetic differences between the 16 different HA subtypes divided into group-1 and group-2. Figure from Influenza hemagglutinin and neuraminidase membrane glycoproteins. J Biol Chem. 2010 Sep 10;285(37):28403-9. Gamblin and Skehel.

The disease pathology of IAV, IBV, and ICV in humans ranges from mild to severe morbidity and mortality, with ICV inducing largely asymptomatic or mild infection and IBV and IAV leading to moderate and severe disease. Influenza is a major public health disease which causes 9.2-35.6 million illnesses; 140,000-710,000 hospitalizations; and 12,000-56,000 deaths annually in the United States [8]. In humans, both IAV and IBV contribute to annual seasonal epidemics, and co-circulation of two different subtypes of IAV, H1N1 and H3N2, and the two distinct lineages of IBV

occurs [9, 10]. Influenza strains typically circulate seasonally within the temperate areas of each hemisphere, while tropical and subtropical areas experience a less defined seasonality of annual epidemics [11, 12]. For seasonal viruses, human immunity from previous infections selects for mutations in the major surface proteins of the virus, HA and NA, leading to “antigenic drift” [13]. The HA serves as a major antigenic determinant due to a high prevalence on the surface of the virus and the ability for the adaptive immune system to generate antibodies against the HA. Notably IAV has the potential to cause pandemics which have occurred four times , beginning with the 1918 (H1N1) influenza pandemic which is estimated to have resulted at least 50 million deaths globally and infection of one third of the world’s population [14, 15]. Additional pandemics have occurred in 1957 (H2N2), 1968 (H3N2), and most recently in 2009 (H1N1) [16-18].

Evolution via Antigenic Drift and Shift

The influenza virus can undergo evolutionary changes through two processes: antigenic shift and antigenic drift. Antigenic shift, described in detail below, occurs via the exchange of gene segments as two different strains infect the same cell, resulting in progeny virus that is distinct from parental virus. Antigenic drift occurs via the accumulation of changes in the antigenic surface proteins through the error prone polymerase and selection in the human population due to pre-existing immunity. Antigenic drift and genetic drift of the non-surface protein segments are the result of incorporation of mutations through error-prone replication with an average of about 1.3 x 10^{-3} to 5.7 x10^{-3} nucleotide changes at each site per year with variation of rates between

segments [19-23]. The HA protein shows the highest level of nucleotide and amino acid substitutions in comparison to the other segments; as immune selection pressure against the HA in hosts leads to a higher rate of ongoing genetic evolution [21]. Many studies show that the HA1 region of the HA evolves at a greater rate than HA2, which is historically more well-conserved. The overall annual nucleotide substitution rate in H3 was found to be 2.3% in HA1 and 1.5% in HA2 without the presence of immune pressure in a plasmid-based expression system. Non-neutral nucleotide substitutions leading to amino acid changes were more frequently seen in the HA1, unlike the HA2 where there was a stronger r to substitutions [24]. One such study compared the areas of high rates of evolution and found that many of the amino acid changes in HA can be attributed to regions which interact with antibodies, for example within the H3 viruses, in the A and B antigenic sites of the HA [13, 25]. Thus, escaping antibody neutralization of the virus can be positively selected for in the immunodominant locations of the HA structure, while lesser rates of change are seen in the areas of the HA important in binding to sialic acid or HA stem regions important for membrane fusion function. Notably, of human influenza subtypes, the H3 lineage of the seasonally circulating strains often undergoes a higher rate of antigenic drift than that of the H1 subtypes which also co-circulate seasonally [26]. In the case of seasonal epidemic subtypes, the HA will continue to undergo antigenic drift at a slower rate which often still results in enough antigenic variation within 1-5 years to result in a need to replace the current vaccine strain targeting that subtype [21]. Ongoing antigenic drift of HA specifically points to the necessity of vaccine updates of the components of the vaccine from

season to season as older vaccine strains becomes less protective with further antigenic variation.

Due to the segmented nature of the genome, the virus is able to reassort by incorporating different segments from two parental viruses and forming a novel virus, a process known as antigenic shift when involving the HA or NA. Incorporation of other segments from different lineages of viruses can lead to enhanced transmissibility or viral fitness in specific hosts and can be seen in some of the events that occurred in the establishment of pandemic viruses in humans [27, 28]. Three of the four pandemics which have been documented in humans have occurred through the introduction of a novel subtype in humans: H1N1, H2N2, and H3N2 [14, 16, 17]. The fourth human pandemic occurred in 2009 and unlike the other pandemics, the H1 and N1 subtypes had previously been seen in humans, emerging as an H1N1 of triple-reassortment from three viral lineages [29]. As each pandemic strain emerged over time the strain would establish as a seasonal IAV strain in humans until the emergence of the next pandemic strain replaced the previous strain. Thus only one IAV strain circulated seasonally in humans, up until 1977 when an accidental release of the previous H1N1 strain allowed the establishment of co-circulation of H1N1 and H3N2 from the 1968 H3N2 pandemic strain. Though the 2009 H1N1 pandemic had the same antigenic subtypes as the circulating seasonal H1N1 virus, due to the distinct nature of the viral antigens from the contemporary H1N1, the pandemic strain was antigenically different enough to result in loss of adaptive immune protection. The triple reassortment classification of H1N1pdm2009 relates to the three distinct lineages from which the various genes originated to emerge as this novel virus. Phylogenetic analysis showed that the triple

reassortant occurred from classical swine, North American avian and human H3N2 lineages. The PA and PB2 genes originating from North American avian strains; PB1 from the H3N2 human strain; and the HA, NP and NS genes of classical swine lineages. After this virus established within swine populations in North America, a second reassortment event occurred with the transfer of the NA and M genes of Eurasian swine viruses, which contributed to the emergence of the A(H1N1)pdm09 [30]. In this case, the HA (H1) from classical swine viruses was more closely related to the 1918 H1, which resulted in some cross-reactivity of antibodies in elderly populations who had previously been exposed to lineages of the 1918 H1N1 virus which circulated until the 1950s [31, 32].

Regardless of the method of genetic change, either through a gradual drift or a quick shift of antigens, the challenges associated with immune evasion of natural or vaccine associated immunity remains. Thus, novel vaccine strategies and antiviral approaches remain a priority for effectively controlling seasonal and pandemic influenza viruses.

Reservoirs and Host

The origin of human influenza A viruses are strains that circulate in wild aquatic avian species [33, 34]. Influenza viruses persist in these natural avian reservoirs which includes avian species from the orders Anseriforms and Charadriiformes in which the 16 HA and 10 NA subtypes are found within species of these two orders. Transmission in these natural aquatic avian species occurs by a fecal to oral route and typically do not cause a significant disease burden on the animal [35]. In aquatic avian species the

preferred host cell receptor for IAV is the α2,3-linked sialic acid which is found throughout the gastrointestinal tract of these avian species [36]. Although aquatic avian species are the natural reservoir, infections and transmission in other avian species experience infection and play a role in transmission events to both other animals and humans in the domestic poultry populations. The distinction of low pathogenicity avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) occurs through the pathogenic effects on galliform fowl species such as chickens. If infection of an influenza strain in young chickens results in a significant level of morbidity and mortality the IAV is classirifed as HPAI. HPAI strains typically include the presence of a polybasic cleavage site that allows for intracellular cleavage of the HA protein so budded virus is already proteolytically activated for fusion. HPAI strains will often result in higher pathogenicity in other species including humans as well as domestic poultry. Infections of LPAI occur largely in the epithelial cells of the gastrointestinal tract of avian species; however, some limited infection occurs in the respiratory tract of avian species as well. Conversely, HPAI strains show a greater level of replication of virus in the respiratory tract in avian species [37]. Though many different IAV transmit within avian species the potential for zoonotic events which infect other species including mammals and more specifically humans, results in an ongoing potential for pandemics.

While transmission of IAV in avian species largely occurs through a fecal-oral route, transmission in mammals such as pigs, humans, and horses occurs via the respiratory route and presents with varying disease severity from mild to severe [38]. Cross species transmission of avian viruses to mammalian hosts such as swine or humans can be determined by several viral and host genetic factors, but receptor

binding is a key determinant. The route of transmission and host range of influenza is often impacted by the preferred receptors for the viruses, which in the case of aquatic avian species are glycans containing α2,3-linked sialic acid found in the gastrointestinal tract, and in mammals, glycans containing α2,6-linked sialic acid found in the respiratory tract [39, 40]. While in humans the respiratory tract is more predominantly lined with the α2,6-linked sialic acids, swine typically have a mixture of both the α2,3- and α2,6-linked sialic acids in the trachea and respiratory tract [41]. Though past speculation often pointed to swine as “mixing vessels” of avian IAV prior to emerging in humans, the IAV strains found in swine populations consist of those that are enzoonotically found in swine and occasional infections of avian and human influenza viruses which either cause a limited outbreak or establish within swine populations which transmit enzonootically. Influenza infection in swine typically results in morbidity but not lor mortality and symptoms include weight loss, nasal discharge, conjunctivitis, fever, and lethargy and viral replication is largely relegated to the respiratory tract. Unlike in human populations, circulation of the viruses in swine populations does not lead to notable antigenic drift. This may be due to a lack of pre-existing immunity in swine populations, which as short-lived agricultural animals are not likely to be re-infected during their lifetimes [42]. The close contact between humans and agricultural swine can result in the transmission of human influenza into swine populations and further reassortment in swine before re-emergence into the human population as a more antigenically distinct virus, as evidenced by the triple reassortant 2009 H1N1 pandemic strain which emerged from swine populations though the subtypes were already established in humans [29].

In addition to agricultural swine and poultry populations which experience influenza infections and sustained transmission within populations, horses are also a animal reservoir which experiences infection by IAV. Influenza infection in horses has been seen with two different subtypes, H7N7 and H3N8, both of which present as respiratory infections which include symptoms such as cough, nasal discharge, loss of appetite and bronchitis [43, 44]. Within horse respiratory tract the sialic acid species include a predominance of α2,3-linked sialic acids, however, H7N7 viruses show a preference of binding to an N-glycoyl sialic acid species as opposed to the more commonly N-acetyl sialic acid species preference seen in avian and mammalian species; yet the linkage preference of binding to the α2,3-linkage is consistent. In contrast, the H3N8 equine influenza has a higher preference for the N-acetyl sialic acid species over N-glycoyl sialic acid; yet still with a preference to α2,3-linkages showing optimizations of the binding of the viruses within the enzoonotic host species [45, 46].

Human respiratory tract contains both α2,3 and α2,6-linked sialic acids with α2,6-linked sialic acids being the predominant sialic acid present throughout, while some α2,3-linked sialic acids can be found in the lower respiratory tract [47]. During introduction of avian influenza viruses into humans, as seen in past pandemics, the preference for sialic acid linkage strengthens towards the α2,6-linkage sialic acids as the virus transmits and sustains within humans [48]. As with other mammalian influenza infection the symptoms of human infection include typical tracheobronchitis and cough seen in other mammalian respiratory infections along with other systemic symptoms which include fever, muscle soreness, chills, and loss of appetite. Replication of the virus occurs in the respiratory tract and reflects the localization of the viral infection

indicated by the preferred sialic acid recpetors throughout the respiratory tract and in the case of the α2,3-linked sialic acids found lower in the respiratory tract.

Viral Life Cycle

As mentioned previously, the virus has a negative-sense segmented genome consisting of 8 segments in both IAV and IBV. These 8 segments can encode up to 13 proteins depending on the strain of the virus. The 8 genes include the trimeric polymerase complex: PB2, PB1, and PA; hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), M (matrix segment), and NS (non-structural segment). The M gene codes for both the M1 and M2 proteins and the NS gene encodes NS1 and NEP proteins through alternative splicing of the gene [49, 50]. Additional accessory proteins are encoded in some IAV including the PB1-F2 which occurs through an alternative open reading frame in the PB1 gene and encodes for a proapoptotic virulence factor [51]. Another example of an additional protein encoded through a frameshift includes the PA-X protein encoded from the PA gene segement yielding a protein which affects the host innate immune response as well as repression of cellular gene expression [52]. An infectious virus particle contains each of these segments which are encapsidated by the NP protein and enveloped by a lipid membrane derived from the host cell membrane through interactions with the M1 scaffolding protein. The lipid membrane contains the surface proteins of the virus: HA, NA, and M2. The HA is important in the initial receptor binding of the virion to the host cell and in fusion between viral and endosomal membranes; while the NA is important in the viral release and dissemination from lipid membrane of the infected cell [53-57]. The M2 protein allows for the influx of

protons from endosomes into the virion interior during viral entry, which initiates the uncoating of the virus gene segments and facilitates their migration to the nucleus where RNA transcription and replication take place [58-60]. HA and NA spikes project from the surface of virus particles, and for most strains HA outnumbers NA on the surface of virion [61-63].

Viral replication requires entry into a host cell which occurs when the HA binds to its receptors, which are glycans terminating in N-acetylneuraminic (sialic) acids found ubiquitously on the surface of host cells. After the virus has attached to host cells, the virus is internalized via clathrin-mediated endocytosis or other mechanisms [64, 65]. Regardless of the entry pathway into the host cell, once in the endosome cellular proton pumps acidify the environment and, at a pH of between 5.0 and 6.0 depending on virus strain, the HA undergoes conformational changes that drive fusion between the host and viral membranes allowing a pore to develop between the viral and endosomal membranes [54, 66, 67]. Following fusion of the viral and endosomal membranes, the viral ribonucleoproteins (vRNPs) are released into the cellular cytoplasm. Also, in the endosome, the M2 protein allows the hydrogen ions to enter the viral complex breaking up the complex of viral envelope with RNPs, and allowing for the free RNPs to be released into the cytoplasm [59]. Uncoating of approximately 30% of the RNP complexes occurs at the point of fusion and blocking of the M2 through ammonium chloride results in a decrease in uncoating efficiency [68].

Nuclear import signals found on a panhandle like structure of the vRNP complex allow for active transport from cytoplasm into the nucleus where mRNA transcription and viral genome replication take place [69]. Influenza transcribes polyadenylated

mRNAs that are primed using 5'-methylated cap structures derived by viral endonuclease cleavage of cellular mRNAs, termed "cap-snatching" [70-74]. Alternative splicing events occur in some of the gene segments resulting in the synthesis and translation of more than one mRNA from some segments as mentioned previously, so that the eight segments of influenza can yield additional proteins [75]. Such splicing of gene segments occurs in segments 7 and 8 of the influenza A genome yielding M1/M2 and NS1/NEP respectively, and for some strains PB1-F2 can be generated in addition to PB1 using an alternative reading frame of segment 2 during translation of this mRNA [76].

The negative-sense vRNA genomes from the virion serve as the template for synthesizing positive-sense mRNA for protein synthesis, or positive-sense cRNA for genome replication. In contrast to the synthesis of mRNA, the production of cRNA results in full-length complementary transcripts of the vRNA without the additions of 5'-cap or polyA tails. Typically, mRNA synthesis is higher than cRNA synthesis in the early stages of infection; however, increasing levels of the proteins important in the replication of the virus, such as NP, may trigger a switch towards increasing cRNA production for further genome replication [77-79]. NP encapsidated cRNAs serve as templates for replication of negative-sense vRNA, which are also encapsidated and associate with the viral RNA polymerase subunits creating new vRNP complexes for nascent virus. The vRNPs associate with matrix M1 protein and nuclear export protein (NEP) for export from nucleus to cytoplasm [80-82]. Following this export from the nucleus, the RNP complexes travel to the apical membrane of polarized epithelial cells where viral surface proteins HA, NA, and M2 have been targeted [83-87].

During the viral replication process, the surface glycoproteins are synthesized by membrane-bound ribosomes and processed within the endoplasmic reticulum and Golgi network where HA and NA are glycosylated and assembled into trimers and tetramers, respectively [88]. The M2 protein is also incorporated into the membrane as a tetramer but is only glycosylated in some virus strains. During transport through the Golgi network, the HA and M2 cysteine residues are palmitoylated [89-91]. During processing through the Golgi network from cis-Golgi to trans-Golgi, HA proteins which have a polybasic cleavage site, as seen in highly pathogenic influenza subtypes, can be proteolytically cleaved by furin-like proteases intracellularly [92]. The presence of this polybasic cleavage site and intracellular cleavage allows the influenza virus to be activated for potential fusion before new virions have even been exposed to the extracellular environment; and such strains often have enhanced pathogenicity within hosts. Subtypes which do not contain a polybasic cleavage site, accounting for the majority of HA subtypes, will be proteolytically cleaved extracellularly by trypsin-like proteases [93].

The transmembrane domains of the surface proteins direct the proteins to the apical surface of the cell and target them to lipid raft microdomains in the membrane, thus providing an area from which budding of new virions will originate [94-97]. The vRNP complexes, in association with the M1 protein, associate near the HA, NA, and M2 proteins at the surface of the cell [98]. The eight unique viral RNPs of influenza must be incorporated to create another infectious virus particle. The genome segments contain specific packaging signals on each of the vRNPs which yield a full complement of the genome in each virion by ensuring the packaging of the eight segments. This is

thought to occur through noncoding and coding regions of the RNA which select for the eight segments necessary for infectious viral particles to associate near the point of budding of the membrane [99-102]. In some cases, productive infection can also occur if two virions carrying less than the full eight segment genome infect the same cell and can produce a complementation of a full infectious particle within the context of co-infection or in local infection of one virus yielding a formation of a full virion following complementation [103]. The ability for co-infection of either incomplete or complete genomes of influenza can allow for reassortant viruses which incorporate different genome segments from parental viruses yielding a progeny comprised of segments from both viruses [104]. These packaging signals may play a role in the level of reassortment which occurs during co-infection with some segments showing preference of packaging signal incorporation between parental viruses and limitations that some segments may have on packaging of other segments [105, 106]. This capacity for the virus to allow for co-infection and further complementation is especially important when understanding the context of interspecies transmission and emergence of novel subtypes during pandemics.

The viral life cycle comes to completion once the segments of the viral genome are incorporated near the plasma membrane where the surface proteins are located, and the curvature of the plasma membrane begins to allow for extrusion, creating an enveloped budding virion. The morphology of the nascent budding virus, either spherical or filamentous, is largely attributed to the interactions and structure of M1 protein [107-110]. To complete viral release, the NA must enzymatically cleave sialic acids located at the viral and cellular surfaces [111, 112]. Since the HA and NA both

interact with sialic acid for opposing functions, binding and release, the activity and specificity of the respective interactions between HA and NA must be balanced to a degree that allows for both sufficient binding and virion release [113].

Hemagglutinin Structure and Functions

The HA trimers extend about 135 angstroms from the surface of the virus and contain a membrane distal globular head domain where the receptor binding site and major antigenic sites are located. A long helical stalk containing a central coiled coil connects the distal head to the membrane anchor [114]. Within the HA stalk domain resides the N-terminus of the HA2 which is a highly conserved, relatively hydrophobic domain known as the fusion peptide because it interacts with the target membrane during the structural rearrangements that lead to fusion. The HA protein is thought to have diverged from the 'common ancestor' HA approximately 3,000 years ago, with further subtype diversification occurring at a higher rate in the last 110 years [20]. The protein continues to mutate and diversify via sequence changes, with sometimes as little as 30% amino acid sequence identity between HA subtypes, however, the proteins themselves still appear structurally similar to one another, shown in Figure 2, and all maintain the ability to function as a class 1 fusion protein [6, 115].

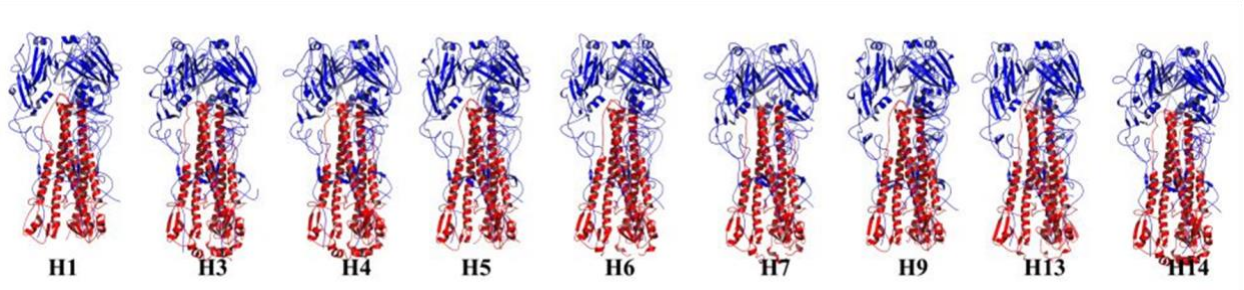

Figure 2. Ribbon structures of multiple HA subtypes that include representatives from both group-1 and group-2.

The HA protein plays two major roles in the viral life cycle: binding of the virus to host cell receptors and the fusion of the viral and endosomal membranes, and these functions are detailed below.

Receptor Binding

HA binds to the host cell receptor, N-acetyl-neuraminic acid, commonly referred to as sialic acid, with preferential binding dependent on the adaptation of the virus to the host species [116]. Typically, human adapted influenza virus HA will bind to α-2,6-linked sialic acid; while avian influenza viruses typically prefer an α-2,3-linked sialic acid[48, 117-119]. The preferences in the HA receptor specificity relate to the difference in glycan receptors present in the host species. There is more α-2,6-linked sialic acid found in the upper respiratory tract of mammals including humans, while avian species; where the virus is enteric in nature, have more α-2,3-linked sialic acid in the gastrointestinal tract.[40, 119-121] Examples of infection with highly pathogenic avian influenza viruses with α2,3-sialic acid recognition show localization to the lower respiratory tract of humans, where there is presumed to be prevalence of the α2,3-linked sialic acid, often leading to severe pneumonia. [47, 122, 123]. As a virus adapts to humans and acquires the ability to successfully transmit from human to human, there are specific mutations near the RBS which are important in the change of sialic acid linkage preference.

The sequence variation noted in the HA subtypes shows that many regions of the HA structure are tolerant to changes whereas other areas of the protein are conserved to varying degrees, including the receptor-binding site (RBS). Structurally the RBS is composed of the 130-loop, 150-loop, 190-helix, and a 220-loop and the most conserved amino acid residues in the site make direct contact with sialic acid or the other sugars in glycan receptors [124-126]. Many surface residues located structurally proximal to the RBS are sites where mutations arise during antigenic drift [55]. Additionally, as influenza strains circulate in humans and are subjected to immune pressure, N-glycosylation sites are added to the trimeric structure and can mask the HA from antibodies [127-129]. Mutations in the RBS can lead to changes in the preference for receptors which is important in changes in host species and transmission. Areas of mutation affecting the RBS include changes in the 190-helix and the 220-loop within the RBS [48, 130-132]. In recent years, antigenic drift of H3 subtype viruses has resulted in a change in the binding preference of the sialyated glycans due to mutations found in the 150-loop, 190-helix, and 220-loop. As a result, more recent viruses are restricted in binding and only recognize longer, branched sialylated and non-sialylated glycans, as well as newly-recognized phosphorylated substrates [133, 134]. The adaptation of avian influenza strains with α2,3-linked sialic acid specificity to human α2,6 specificity also relate to mutations which occur in the receptor binding site. Certain residues are found to be highly important for host specificity and include E190D and G225D in H1 subtypes and G226L and G228S in H2 and H3 subtypes [53, 135-138]. These residues have been shown to be critical to the ability for novel strains to cause pandemics in humans as mutations here are hallmarks of host adaptation altering the specificity of

receptor recognition to ensure successful human to human transmission [48]. The HPAI subtypes such as H5N1, H7N9 and H9N2 have some residues which have been identified in host adaptation and transmission from avian species to humans, which include Q226L in H5N1 when combined with N224K and N158D amino acid substitutions, and G228S in H7N9 which already contains a 226 Leucine residue seen in H5N1 [139-143]. In some cases, these residue substitutions result in increased affinity for the human α2,6-linked receptor binding such as with 226 and 228 residues, while others may play a role in specificity by reducing binding affinity for the avian α2,3-linked sialic acids as found in the E190D substitution. As shown in Figure 3, the interactions between analogs of the human and avian receptors show dynamic interactions within the 130 and 220 loops of the HA RBS.

Other substitutions affecting the RBS include those amino acid mutations which disrupt the glycosylation sites on the HA1 which allow increased binding to the α2,6-linked sialic acids when in conjunction with the Q226L and N224K mutations. Mutation of the N158 and T160 residues of the globular head HA1 cause the loss of glycosylation sites and enable increased binding to α2,6-linked sialic acid receptors. These mutations show increased occurrence in highly pathogenic avian influenza strains [138, 144].

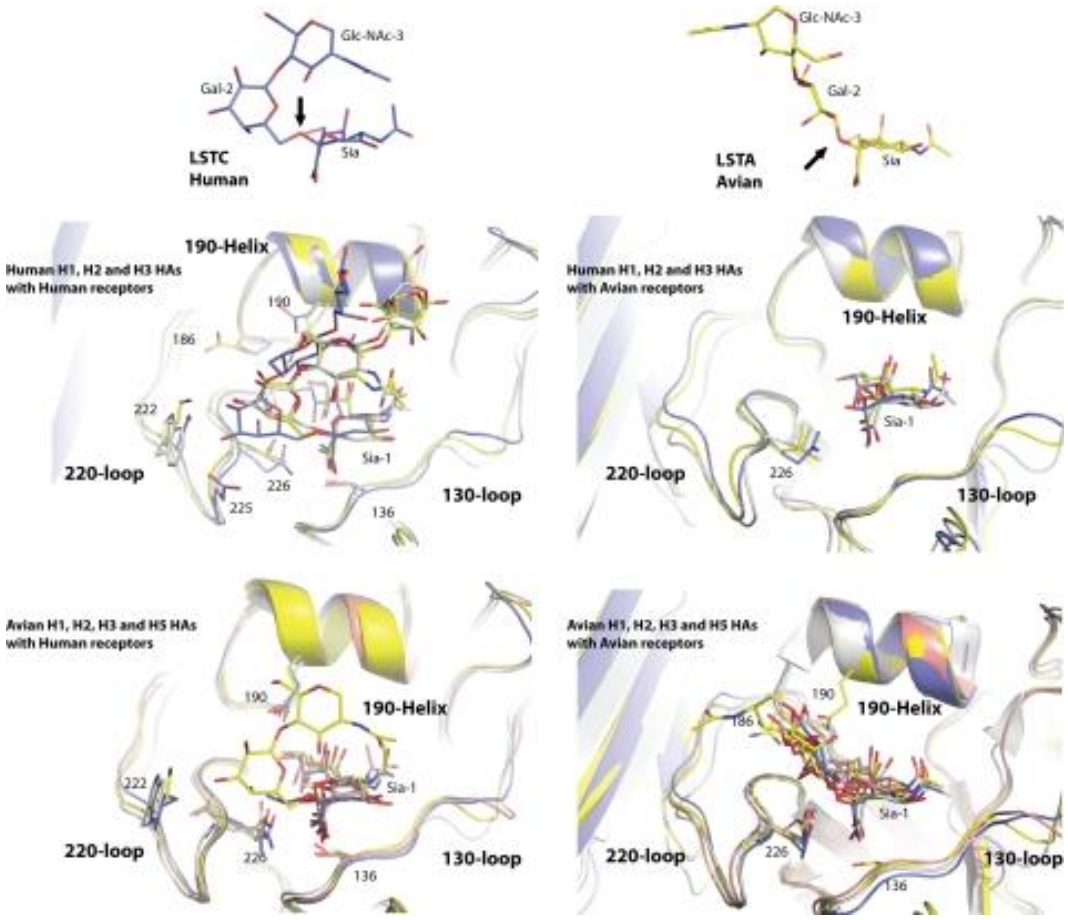

Figure 3. Top panel shows the analogs of human (left) and avian (right) cell receptors recognized by the HA. Middle panel shows interaction of the human HA RBS with the human (left) and avian (right) analogs. Bottom panel overalys the avian HA interaction with human (left) and avian (right) receptor analogs. Image from Gamblin and Skehel. J Biol Chem. 2010 Sep 10;285(37):28403-9. doi: 10.1074/jbc.R110.129809. Epub 2010 Jun 10.

Membrane fusion

Proteolytic activation of fusion potential

The HA is translated, oligomerized, and glycosylated, as an uncleaved protein referred to as HA0. For most viral strains, each monomer of the uncleaved HA0 is cleaved by extracellular proteases resulting in the disulfide-linked subunits, HA1 and

HA2[114, 145]. This cleavage event activates fusion potential for two reasons. First, the cleavage liberates the HA2 N-terminal fusion peptide domain. Second, the positively-charged N-terminus of the newly-formed fusion peptide is buried within a negatively-charged fusion peptide pocket, which transitions the HA into a metastable structure that can subsequently be triggered into structural rearrangements by acidification of endosomes [145, 146]. In human and low pathogenic avian influenza (LPAI), the cleavage of HA0 occurs at a single arginine residue between HA1 and HA2 by trypsin-like proteases, while in HPAI viruses the presence of a polybasic cleavage recognition sequence, R-X-R/K-R allows for intracellular cleavage of the nascent HA in the trans-Golgi by furin-like proteases, resulting in the presence of a fusion-activated HA on the surface of nascent virus particles, as found in pathogenic strains of H5 and H7 subtype viruses [147-150].

Acid-induced conformational changes and membrane fusion

The binding of the HA to the cell allows for internalization either by clathrin-mediated or independent endocytosis [64, 65]. As endosomal trafficking progresses, the environment becomes increasingly acidic starting from pH 6.0-6.5 in the early endosome to closer to pH 5.0-5.5 in the later endosomes and finally as low as pH 4.6 to 5.0 in lysosomes. Each virus strain has an optimal pH at which fusion initiates in the endosome [65, 151, 152]. Conformational changes have also been shown to be triggered by increasing temperature at neutral pH *in vitro*, and the temperature at which this occurs is directly proportional to the pH of conformational change, ie. more stable strains that fuse membranes at lower pH require higher temperature to trigger structural

changes [153]. This, and the fact that the low pH structure is stable to temperatures significantly higher than the neutral pH structure provides further evidence that the

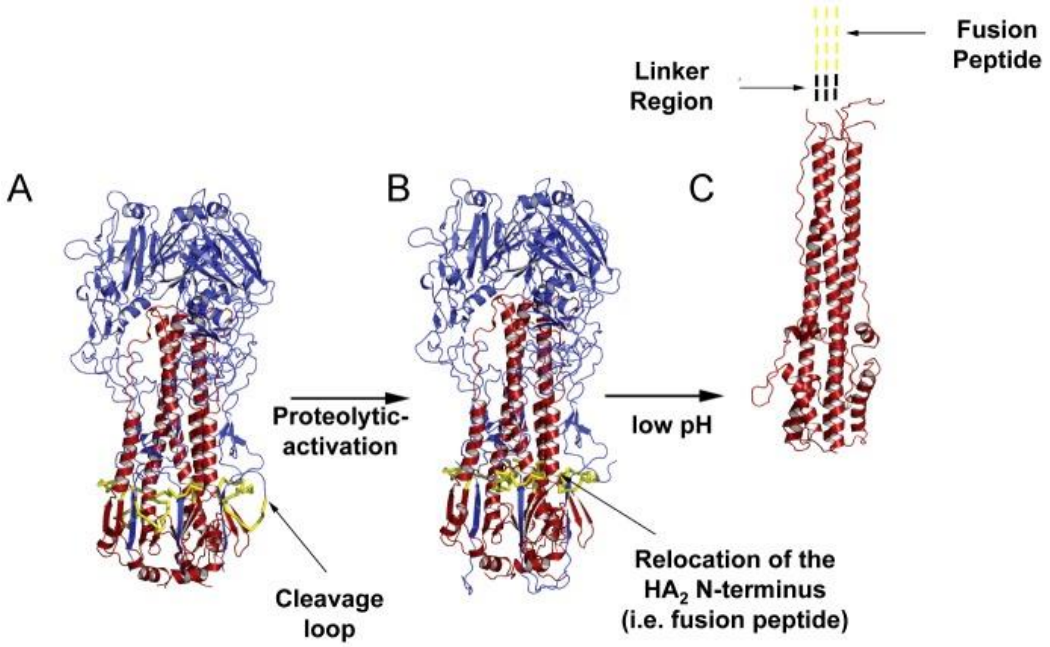

Figure 4. (A) Trimeric structure of the HA protein prior to proteolytic activation via cleavage. (B). Postiviely-charged N-terminus of the fusion peptide relocates to a negatively charged fusion peptide pocket. (C) Extrusion of the fusion peptide and loop to helix change extends the fusion peptide towards the endosomal membrane. From Galloway PLoS Pathog. 2013 Feb;9(2):e1003151

neutral pH conformation of HA is a metastable structure [154, 155]. Residues throughout the HA have been shown to affect pH of fusion, and these reside at structural interfaces between domains that relocate as a result of acid-induced conformational changes [55, 66, 156-161]. The irreversible structural transitions that convert the metastable neutral pH conformation of HA to the more stable structure of the low pH conformation involve several HA domains. The membrane distal head domains detrimerize and separate from the stalk domains. The fusion peptide located at the N-terminus of the HA2 subunit is extruded from its buried location in the trimer interior as shown in Figure 4. The short HA2 helix and extended polypeptide chain that connect it to the central coiled coil, all become helical, and as a result, the coiled coil is

extended and relocates the fusion peptide into the endosomal membrane. In addition, residues of the long coiled coil helices proximal to the fusion peptide in the neutral pH structure undergo a helix-to-loop transition and reorient all residues C-terminal to this by 180 degrees. This effectively “jackknifes” the structure and allows the viral and endosomal membrane to meet at a “contact zone”, consisting of 15-20 HA proteins and the target endosomal membrane [162].

A number of studies indicate that residues located near the hydrophobic N-terminal fusion peptide are particularly important for HA stability [163]. Highly conserved residues 112 of HA2 and 17 of HA1 of the H3 group-2 HA subtype are both buried by the HA2 N-terminal fusion peptide residues in neutral pH conformation and have been identified as being important in stabilizing the fusion peptide region. In the well-studied X-31 (H3N2) influenza strain, mutations of the wild-type aspartic acid (Asp) of HA2 112 and the histidine (His) of HA1 17 result in destabilizing effects through the disruption of hydrogen bonding to other amino acids within the first six residues of the N-terminal region of the HA2 [159]. The first ten residues of the fusion peptide as well as several residues located within the fusion peptide pocket are found to be highly conserved between subtypes, and these are some of the most structurally rearranged parts of the HA stem region [164]. Numerous studies on amino acid substitution mutants, as well as deletion and insertion mutations in the fusion peptide, provide evidence that this region is critical for the overall stability of neutral pH HA [159, 161, 165-170]. Some residues are conserved within all of the HA subtypes, excluding the bat H17 and H18, and others are conserved within the two groups of HA, group-1 and group-2. Residues in the fusion peptide region that are conserved in all subtypes

include K51, D109 and D112 all of the HA2 subunit. Residues conserved by groups include the HA1 17 residue and residues 106 and 111 of HA2. Within the group-1 subtypes, the 17 residue is conserved as a tyrosine, while the 106 residue is either an arginine or lysine and the 111 residue a histidine [161, 171]. Many of these residues appear to be critical to the conformational change due to the ability for these amino acids, such as histidine, to be protonated during the low pH conditions of the late endosomes. Ionizable residues found within the HA fusion peptide pocket include the His 17, Arg 220 and Arg 229 of HA1 along with the His 111, Asp109, and Asp112 of HA2 residues of HA2 [172] [145]. As such, experimental mutations at these residues can yield altered pH of fusion or disruption of fusion.

A number of mutational studies of the HA of multiple subtypes have shown that conserved HA2 residue K58I mutations resulted in decreased pH of fusion in stabilized HAs while the D112G mutations at this conserved HA2 residue resulted in an elevated pH of fusion across multiple subtypes [169]. Studies using double mutants that contain destabilizing mutations such as D112G and stabilizing mutations like K58I from different regions of the HA, show that mutations proximal to the fusion peptide have the dominant effect on overall stability phenotype [173]. Furthermore, studies on the kinetics of structural changes using conformation-specific monoclonal antibodies that target different regions of the HA indicate that structural rearrangements in the stem region precede the unfolding of membrane distal head domains [174]. Studies on the fusion kinetics of single virions determined that withdrawl of the fusion peptide from the pocket is the rate-limiting step for conformational changes [175]. Similarly, studies using hydrogen-deuterium exchange and mass spectrometry to analyze the dynamics of acid-

induced changes concluded that extrusion of the fusion peptide precedes other aspects of the overall structural rearrangements [67]. Based upon these studies, we embarked on mutagenesis studies on various subtypes and strains of group-1 HAs in order to identify residues that might play a significant role in the initiation of the membrane fusion process, and the results of these are detailed in Chapter 2 of this thesis.

Antibodies and Fusion

Monoclonal antibodies have been employed to understand the structure of the HA, with antibodies generated which can detect either neutral pH pre-conformational change HA or a post-conformational change HA, dependent on exposure of epitopes in the low pH conformation [176]. The antigenic sites targeted by many of these antibodies have been mapped by selection of antibody-resistant mutants or X-ray crystallography of HA:Aby complexes (Knossow and Skehel, 2006) Antibodies against H3 X-31, such as HC31, HC67, and HC263 are unable to react with low pH HA as they recognize the trimeric interface of head domains that dissociate upon acidification; while antibodies capable of reacting with areas located on site A, a loop structure on the HA head which is not altered at low pH, would maintain recognition at both neutral and low pH conditions of the HA [177-179]. Similarly in H2, additional antibodies have been generated that are HA conformation-specific, including the monoclonal antibody 2/9 which recognizes the low pH structure of the H2 subtype and other subtypes within group-1 [180]. These conformation specific antibodies are critical to being able to interrogate the status of HA structural rearrangement. These reagents are frequently used in assays to verify that a post fusion epitope is exposed appropriately following

cleavage and exposure to low pH conditions ensuring that the influenza HA is capable of undergoing the necessary conformational changes required for fusion.

The process of fusion can be inhibited by the binding of antibodies which prevent the low pH induced structural rearrangement of HA [181]. Some antibodies have been shown to bind to the HA1 head domains of two monomers, thus locking the globular head domains like a clamp over the HA stalk [182]. However many of the antibodies which are broadly neutralizing, target the more conserved stem region of the HA which is largely comprised of the HA2 domain [183, 184]. In recent years, more interest has focused on understanding how to generate and elicit broader immune responses to influenza infections by broader recognition of multiple subtypes and also through efforts focusing on inhibiting the fusion mechanism.

Biological Impacts of the pH of Fusion

Regardless of host or subtype, the HA protein sequence and structure can vary to an extent without disrupting receptor binding function and the ability to be activated and undergo the large conformational rearrangements for fusion. As mentioned above, the conformational change of HA is irreversible, and as such, must occur at an appropriate time: once the virus is internalized by the host cell endosome and is exposed to a sufficiently low pH. The HA protein must exhibit an optimal pH to allow the HA to be stable enough within the environment during transmission, but labile enough that when in the endosome, it is able to undergo the conformational change necessary for fusion at an optimal time. The pH of HA activation can range from pH 4.8-6.2 depending on the viral strain [152, 185]. Typically the influenza strains infecting avian

and swine species have a higher pH of fusion activation (a less stable HA) [186-188], whereas, strains that have circulated and transmitted in humans typically have lower pH of activation [152, 189]. This work suggests that as adaptation occurs in humans the average pH of fusion decreases, as seen in the early to late isolates of the 2009 pandemic where initially pH of fusion was 5.5-5.6 and, as transmission continued, the average pH of fusion ranged from 5.2-5.4. Swine viral isolates have been shown to have a broader range of pH of fusion (5.5-5.9) which consequently allows for sufficient overlap with both avian and human-adapted influenza viruses[188, 189]. The increased stability of HA allows the human-adapted strains to be stable in the slightly acidic environment (pH 5.5-6.5) of the human nasal cavity and prevents conformational changes of the HA from occurring before the more acidic late endosome environment facilitating the viral release in host cells[190]. Since the conformational change that the HA undergoes at low pH is irreversible, if the HA is exposed to environment conditions outside of the host cell sufficient to cause the HA to undergo the irreversible change prematurely, the virion will be inactivated. In humans the infection of influenza occurs and transmits through the respiratory tract, in avian species and especially in the natural reservoir of aquatic avian species, the transmission and infection is through the fecal oral route typically through indirect exposure to contaminated water habitats, thus influenza circulating in avian species has been shown to persist longer in more basic pH conditions (7.2-7.5), cooler temperatures, and less salinity [191].

Additional studies have shown that residues stabilizing the HA can increase the stability of the virus to allow for efficient airborne transmission or gain the ability to transmit in mammalian hosts, as demonstrated by H5N1 HPAI gain of function studies

[143]. Similarly, swine infection of the 2009 pandemic influenza strain allowed for appropriate receptor binding and HA stabilizing mutation adaptations to increase human transmission, allowing the classical swine virus to mutate and increase transmission potential [192, 193]. The changes in HA that affect the pH of fusion are clearly seen to be important in the establishment of viruses within new hosts and the emergence of pandemic viruses as they adapt to successfully transmit between hosts. Understanding HA and the mechanism by which specific domains of the protein influence its major functions, will lead to opportunities for better vaccine development and strategies to target the HA and control the virus.

CONSERVED HISTIDINE IN GROUP-1 INFLUENZA SUBTYPE HEMAGGLUTININS IS ESSENTIAL FOR MEMBRANE FUSION ACTIVITY

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ABSTRACT

Influenza A viruses enter host cells through the endocytic pathway, where acidification triggers conformational changes of the viral hemagglutinin (HA) to drive membrane fusion. The HA structural rearrangements are well documented, but the molecular basis for initiating the fusion process are not explicitly defined. Extrusion of the fusion peptide from its buried position in the neutral pH structure is critical, and several lines of evidence suggest a role for conserved ionizable residues that are buried by the fusion peptide following proteolytic activation. Here we report mutagenesis studies on expressed HAs representing several Group-1 HA subtypes containing substitutions for a highly-conserved histidine at HA2 position 111. Analysis of cell surface expression, antigenic characterization, and membrane fusion activity reveal that His 111 is essential for HA functional activity of these subtypes and support continued efforts to target this region of the HA structure for vaccination strategies and the design of antiviral compounds.

1. Introduction

Influenza A viruses impose significant healthcare and economic burdens through seasonally recurring epidemics, and the threat of antigenically novel pandemic strains emerging in the human population is an ongoing concern. Vaccination remains the best option for preventing seasonal illness, but coverage rates are not ideal and on occasion, vaccine candidate mismatch or suboptimal efficacy cause problems with particular strains. Influenza antivirals currently licensed in the United States include the ∝-adamantane M2 inhibitors, NA inhibitors, and the recently approved inhibitor of the cap-dependent endonuclease activity of the PA polymerase subunit. These generally need to be administered shortly after onset of symptoms to alleviate illness, and the selection of drug resistant strains is a concern for all three classes of inhibitor [194-196]. With such limitations, there continues to be a need for the design of both new vaccine candidates and novel antiviral compounds against seasonal human influenza viruses and other strains that continue to circulate in avian and animal reservoirs.

Though no anti-HA drugs for influenza have been FDA approved to date, inhibitors of membrane fusion have been utilized with varying degrees of success for viruses including members of the *Retroviridae* and *Paramyxoviridae* families. Since the early 1990s, several strategies designed to block HA fusion function include the use of compounds that inhibit the acid-induced conformational changes required for fusion or trigger these structural rearrangements prematurely [197-199]. The capacity to block HA conformational changes has been proposed as a potential mechanism of action for anti-HA stem antibodies, which have been touted as “universal vaccine” candidates

[183, 184, 198, 200-203]. Recently, a compound based on structural data of antibody:HA complexes was shown to bind to the HA stem of Group-1 HA subtypes of influenza A viruses to inhibit acid-induced conformational changes, and antiviral activity was observed in mice following oral administration [204]. However, the experience gained from the use of other antivirals for influenza suggests that the propensity for selection of resistant mutants creates potential roadblocks for broadly effective vaccines or antivirals acting on HA. With this in mind, we sought to identify regions of the HA that are involved in the initial triggering of conformational changes as endosomes are acidified and define particular residues that are conserved or immutable without debilitating function.

The HA, a type I membrane glycoprotein, is synthesized as precursor polypeptides (HA0) of approximately 550 amino acids that associate non-covalently to form a homotrimer. Each monomer of the HA0 trimer requires proteolytic cleavage into the disulfide-linked subunits HA1 and HA2 in order to activate virus infectivity [93, 205]. Following attachment of virions to host cells and internalization, the acidification of endosomes triggers irreversible conformational changes in the HA that drive the fusion of viral and endosomal membranes, releasing the viral genome into the cytoplasm. For the HA of the A/Aichi/2/68 (H3N2), high resolution structural information is available for HA0, neutral pH cleaved HA, and the low pH conformation adopted following acidification [114, 145, 206, 207]. The structure of the HA0 trimer reveals that the stem region contains a surface loop domain in each monomer that is oriented to expose the conserved arginine residue that is cleaved by activating proteases. Upon cleavage, the membrane proximal portion of the loop becomes the N-terminus of the HA2 subunit,

and this conserved hydrophobic domain is commonly referred to as the fusion peptide. The fusion peptide then relocates into a cavity lined by ionizable residues, to orient into the interior of the cleaved neutral pH HA structure. The post-cleavage structure is metastable and is now “primed” for fusion activity. Subsequent conformational changes induced by acidification of endosomes convert the HA into highly stable helical rod structures that bring viral and endosomal membranes together in the fusion process.

Although the structure of the HA0 cleavage site for H1 subtypes appear to differ from other subtypes [145, 208, 209], all subtypes relocate their fusion peptides into the trimer interior upon cleavage, burying ionizable residues [114, 126, 146, 210, 211]. These include HA2 residues Asp109 and Asp112, which are completely conserved across all HA subtypes of influenza A viruses and form a network of hydrogen bonds with the newly relocated fusion peptide. HA2 Lys 51 is also conserved across all subtypes, whereas residues at positions HA1 17, HA2 106, and HA2 111 are reasonably well conserved in group-specific fashion. In Group-1 HA subtypes, the amino acids residing at these positions are Tyr, Arg/Lys, and His, respectively, and in Group-2 HAs they are His, His, Thr/Ala. Numerous studies show that mutation of residues in and around the fusion peptide can influence HA stability, and the HA of Aichi has been particularly well characterized [159, 161, 170, 212-214]. For this Group-2 HA, most of the mutations in this region destabilize the HA structure and cause it to mediate membrane fusion at elevated pH. However, mutation of HA1 residue 17 from His to Tyr, which is present in Group-1 HAs, leads to a significant decrease in the pH at which conformational changes and membrane fusion take place. The His residue at HA1 position 17 makes hydrogen bonds to carbonyl oxygens of fusion peptide residues 6

and 10 via a water molecule, and the results suggest that protonation of this residue may contribute to the initiation of conformational changes required for fusion activity. In Group-1 HAs, the larger side chain of the conserved Tyr at HA1 position 17 allows for formation of hydrogen bonds directly to fusion peptide residues 10 and 12, and presumably, analogous hydrogen bonds stabilize the structure of the HA1 H17Y mutant of Aichi HA. If the Tyr at HA1 position 17 has a stabilizing effect on the neutral pH structure of Group-1 HAs, we postulated that other residues in this region might be involved in destabilizing such HAs during acidification of endosomes. Therefore, we targeted HA2 residue 111 as a potential residue of interest, as it is invariant in all naturally occuring isolates of Group-1 HA subtypes. Here we report studies on expressed HAs of several Group-1 subtype viruses, as well as a selection of Group-2 HAs, to examine the phenotypes displayed by mutants with changes at HA2 position 111, as well as HA1 17, with respect to membrane fusion activity and the pH at which it is mediated. We show that, for all HAs examined here, substitutions at this position resulted in a loss of fusion activity, suggesting a critical role for this residue.

2. Materials and methods

2.1 Cells and antibodies

Vero cells (CCL-81) were obtained from ATCC and were used for transfections for the following assays: a luciferase-based reporter assay to assay fusion and western

blots and radioactive labeling and immunoprecipitation to assay cleavage and surface expression. BSR-T7/5 cells (originally obtained from Karl-Klauss Conzelmann) served as the target cells in the luciferase-reporter based assay. For the syncytia formation assay, BHK-21 cells (ATCC CCL-10) were transfected with HA-plasmids to analyze fusion, described below.

The HA specific antibodies utilized in this study were described previously [152, 169]. BEI Resources provided the following: goat anti-H1, goat anti-$H2_{Singapore}$, goat anti-H3, goat anti-H10, goat anti-H11, goat anti-$H1_{PR8}$, goat anti-$H1_{Cal}$, goat anti-$H5_{VN}$. Aichi HA was detected using rabbit anti-X31 serum.

2.2 Plasmids

The wild-type HA expression plasmids have been described previously [152, 169]. To introduce the Y17H, H17Y, H111A, and T111H mutations, the wild-type HAs were PCR amplified from the expression plasmids and cloned into pCR-BLUNT, according to the manufacturer's instructions. The pCR-BLUNT plasmids containing the HA gene of interest were modified using QuikChange site-directed mutagenesis to incorporate each mutation. Ligation independent cloning (LIC) procedures were used for the generation of the final plasmids. For LIC, the mutant HA genes in the sequence-verified pCR-BLUNT clones were amplified using LIC specific primers: a + sense with the 5' flanking sequence 5'TACTTCCAATCCATTTGCCACCATG, and a (-) sense primer with the 5' flanking sequence of 5'TTATCCACTTCCATTTCTCA. Standard LIC

procedures described previously [152, 169] were used for the generation of each of the WT and mutant plasmids.

2.3 Western blot assay

For gel analysis, 40 μl of each sample was resolved by SDS–10% PAGE and transferred to nitrocellulose membrane. After being blocked in Tris-buffered saline (TBS) containing 0.1% Tween 20 (v/v) and 5% non-fat milk (w/v) at 4 °C overnight, the membranes were rinsed and incubated at room temperature for 2 h with different anti-HA antibodies. The membranes were washed and incubated with AP-labeled protein A/G (Thermo Fisher Scientific, MA, USA) at RT for 2 h. The protein bands were then visualized by incubating with the developing solution (p-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate toluidine (BCIP)) at RT for 15 min. The relative intensity of the bands corresponding to HA0, HA1, or HA2 was determined using ImageJ (NIH) v.1.33u (USA). The percent cleavage was determined by using the equation [$HA_1/(HA_1+HA_0)$] x100, and the percent cleavage relative to the wild type was calculated using the equation [(% cleavage mutant HA/% cleavage of WT) x 100].

2.4 Radioactive labeling and Immunoprecipitation

Plasmids (1 μg) containing the WT or mutant HA was complexed with Plus reagent and transfected using Lipofectamine (Invitrogen) into Vero cells. After an incubation of 16-18 hours, the cells, now expressing HA, were washed 2x with PBS and

incubated at 37°C in starvation media, MEM (-Met –Cys), for 30 min. A 15 min pulse with 25 uCi of [^{35}S]-methionine (Perkin Elmer) followed, after which the cells were washed with PBS and incubated at 37°C in complete media with excess (20x) cold methionine for a 3 hour chase period. To assay for cleavage, the cells were washed once with PBS and incubated in DMEM or DMEM plus 5ug/ml TPCK trypsin (Sigma) at 37°C for 30 minutes. For the examination of surface expression, surface proteins were biotinylated using 2 mg EZ-link Sulfo-NHS-SS-Biotin (Thermo Scientific) in PBS pH 8.0 for an incubation time of 30 minutes at ambient temperature. After incubation, biotinylated samples were washed with 50mM glycine in PBS, pH 8.0 to quench the excess biotin. Cells were washed a final time with PBS and were lysed by the addition of 0.5ml RIPA buffer (50mM Tris-HCl, pH 7.4; 150mM NaCl; 1mM EDTA; 0.5% deoxycholate; 1% triton X-100; 0.1% SDS). A 10 minute centrifugation at 16,000x g pelleted the cell debris to leave cleared lysates remaining.

For the antibody pull-down, HA specific antibodies were conjugated to Protein G dynabeads (Invitrogen) in PBS + 0.02% Tween-20 for an hour at room temperature. Unbound antibody was removed following a wash step with PBS+ 0.02% Tween-20. The lysates were also pre-cleared using the Protein G dynabeads for an hour at room temperature. The Protein G/ antibody complexed beads were incubated with the pre-cleared lysates for an hour at room temperature. Following incubation, the beads complexed now with the specific HAs were washed three times with PBS and resuspended in 30μl 1x SDS buffer. A 5 min boil and brief centrifugation step removes the proteins from the beads.

For the precipitation of biotinylated proteins, the beads complexed with Protein-G/antibody/antigen were washed 3 times with PBS and resuspended in 50μl 50mM Tris-HCl+0.5% SDS. The beads were boiled for 5 minutes and centrifuged to dissociate the proteins, so that they may be collected. 25μl of the eluate is used for a subsequent pull down of the biotinylated proteins using Streptavidin M-280 Dynabeads (Invitrogen) to isolate and precipitate cell-surface expressed HA. 100μl of beads per sample were washed three times with PBS + 0.2% BSA and then resuspended in 975μl streptavidin binding buffer (20mM Tris-HCl, pH8.0; 150mM NaCl; 5mM EDTA; 1% Triton X-100; 0.2% BSA). The eluate (25μl) was added to the beads in the streptavidin binding buffer and incubated for 1 hour at room temperature. The beads were then washed with PBS+ 0.2% BSA to remove unbound substrates and resuspended in 35μl 1x SDS sample buffer. The beads were boiled for 5 min and centrifuged to collect the dissociated proteins. The remaining 25μl of the immunoprecipitation elution was used to determine the total amount of HA protein in the sample. 10μl of 3x SDS sample buffer was added to the total HA sample for a final volume of 35μl.

For gel analysis, 18μl of samples were loaded and run on 12% pre-cast SDS- PAGE gels. The gels were fixed with 50% methanol/10% acetic acid solution at room temperature for 30 minutes and dried under a vacuum for 1 hour at 80°C. Once dried, the gels were stored in a cassette for exposure to a phosphorimaging screen. The Phosphor screens were scanned on a Typhoon Trio variable mode imager (GE Healthcare Life Sciences). ImageQuant software was used to determine the intensity of the HA0, HA1 and HA2 bands. The values were normalized to methionine content of the appropriate subunit. Percent cleavage was determined using the equation

(Ha2/(Ha2+Ha0))x100% and the percent cleavage relative to wild-type calculated as ((% cleavage mutant HA/% cleavage of WT)x100). To examine surface expressed HA and for the comparison of wild-type and mutant levels, the total level of HA in each lane was quantified and the amount of surface expressed HA was determined by the following equation, (cell surface HA/ total HA)x100 and then the percent relative to wild-type surface expression was calculated as ((% surface expression mutant HA/% surface expression WT HA)x100).

2.5 Fusion Assays

Membrane fusion mediated by the HA proteins was measured in 2 assays: a qualitative syncytia assay and a quantitative luciferase reporter based assay. For the syncytia assay, BHK-21 cells were transfected with 1μg of HA containing plasmids complexed with Plus reagent and Lipofectamine (Invitrogen) per manufacturer's instructions. 24 hours post-transfection, the cells were washed with PBS and were treated with serum-free DMEM plus 5μg/μl TPCK-trypsin (Sigma) for 15 min at 37°C. The trypsin containing media was removed and the cells were washed with PBS and then exposed to low pH PBS, adjusted with 100mM citric acid, for 5 min. The low pH was neutralized by the removal of the PBS and the immediate addition of complete growth media (CGM). The cells were incubated in CGM for 2 hours at 37°C and then were fixed and stained using a Hema3Stat Pak (Fisher Scientific) for visualization. Syncytia formation was imaged and recorded using a Zeiss Axio Observer inverted microscope with attached digital camera.

For the quantitative luciferase-based reporter assay, 1μg HA-plasmid and 1μg of plasmid containing the gene for firefly luciferase under a T7 bacteriophage promoter were complexed with Plus reagent and transfected into Vero cells using Lipofectamine (Invitrogen). 16-18 hours after transfection, the cells were washed with PBS and treated with 5μg/μl TPCK trypsin in serum-free DMEM for 30 minutes. The trypsin treatment was followed by a wash and a 30 minute incubation in serum-free DMEM plus 20μg/μl trypsin inhibitor to neutralize any remaining trypsin activity. BSR-T7/5 cells, which constitutively express bacteriophage T7 RNA polymerase, were overlaid on to the Vero cells expressing HA at the cell surface. The combined cell populations were incubated for 1 hour at 37°C to allow for the BSR-T7/5 cells to adhere to the existing monolayer of Vero cells. Non-associated cells were removed via a gentle PBS wash. The combined monolayer was exposed to low pH PBS, adjusted with 100mM citric acid, and incubated for 5 minutes at 37°C. The acidified PBS was removed, and the low pH environment was neutralized by a wash and then the addition of complete growth media. The combined cell populations were incubated at 37°C in the CGM for 6 hours to allow for fusion and the transfer of the T7 polymerase and expression of the T7-luciferase plasmid. The cells were then washed with PBS and lysed with 0.5ml of Reporter Lysis Buffer (Promega). The lysates were frozen for 16-18 hours and then collected. Cell debris were removed following centrifugation at 15,000 x g at 4°C and 150μl of the clarified lysate was transferred to a white, flat bottom, polystyrene 96-well plate (Corning). 50μl of luciferase assay substrate (Promega) was injected into each well and luciferase activity, indicating a successful fusion event between the two cell populations, was quantified using a BioTek Synergy 2 Luminometer.

2.6 Enzyme-linked Immunosorbent Assay

Surface expression of the HA, as either the pre- or post- conformational change structure, was determined by Enzyme-linked Immunosorbent Assay (ELISA). 0.1μg/well of plasmid containing the WT or mutant HA was complexed with Plus reagent and transfected using Lipofectamine (Invitrogen) into Vero cells in a 96-well plate. After an incubation of 16-18 hours, the cells, now expressing HA, were washed with PBS, treated with 5μg/μl TPCK trypsin diluted in serum free DMEM for 10 min at 37C. Following trypsin treatment, cells were washed with PBS and incubated with pH-adjusted PBS, using 100 mM citric acid, either at a neutral pH 7.0 or a low pH for 5 min at 37 C. Following pH treatment, wells were incubated with a panel of antibodies, including a polyclonal A/Singapore/57 from BEI Resources as well as 2/9, an H2 monoclonal antibody specific to a low pH conformation. Antibodies were diluted in 2% percent BSA and incubated in the wells for 1 hour at 37C. Following the primary antibody incubation, the wells were washed 3x with PBS-Tween (0.05%). An appropriate HRP-conjugated antibody, donkey anti-goat HRP conjugated antibody or goat anti-mouse HRP-conjugated antibody were used for the H2 polyclonal and conformation specific 2/9 antibody, respectively, which was diluted in 2% BSA and added to the wells for 1 hour at RT, while rocking. Following, wells were washed again 3x with PBS-Tween (0.05%) before the addition of 50µl TMB Substrate where the reaction proceeded for 5 min before being stopped by the addition of 150µl of TMB Stop

solution. The optical density (O.D.) of each well was measured at 450 nm using a spectrophotometer.

3. Results

3.1 Rationale and structural locations of critical residues

Phylogenetic and structural analyses of the hemagglutinins of the 16 HA subtypes found in avian reservoirs show that they can be divided into five clades that segregate into two groups, Group-1 and Group-2 [6, 7, 146]. A number of structural differences distinguish the two groups, including the following: 1) compared to Group-2 HAs, the short helix of the HA2 hairpin loop of Group-1 HAs is extended by a half turn and the peptide chain that links the two helices has a “higher” turn near the top of the long helix. 2) The membrane-distal heads of Group-1 HAs, therefore, ride “higher” on the stem domains, and are rotated relative to group-2 HAs stem domains along the three-fold axes. 3) Several residues in the region of the fusion peptide segregate in group-specific fashion, and notably, HA1 position 17 and HA2 positions 106 and 111 are conserved as Tyr, Arg, His in Group-1 and His, His, Thr in Group-2 HAs, respectively. This region in the vicinity of the fusion peptide forms the focus of our current studies, and the structural locations of the residues of interest are shown in Figure 1. Previous work by our lab and others suggests that residues in the stem region in general, and in the fusion peptide region in particular, may be critical for providing

initial triggers for the acid-induced structural rearrangements required for membrane fusion activity [67, 145, 146, 161, 173-175, 215].

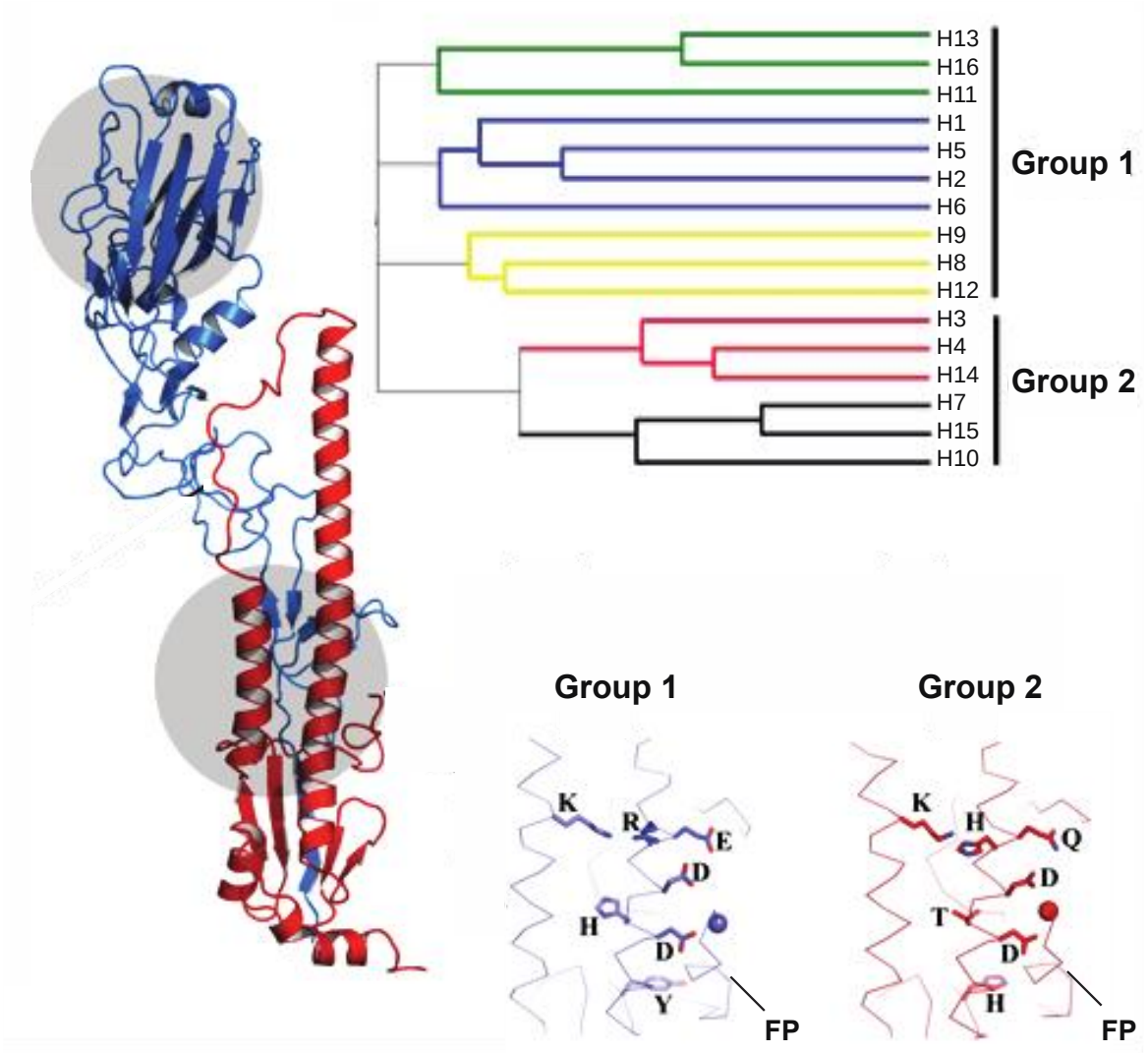

Figure 1. (A) The H3 subtype monomer is shown with the HA1 displayed in blue, and HA2 shown in red. The gray circles highlight the receptor binding domain (top) and fusion peptide pocket (bottom). The structural locations of the HA1 17 and HA2 111 residues are indicated by arrows. The lower right of the panel shows the residues specific to group-1 and group-2 clades which are highly conserved within each group. The HA1 17 is conserved as Tyr in group-1 and His in group-2, while HA2 111 is conserved by group as His and Thr in group-1 and group-2 respectively. The HA2 N-terminal fusion peptide is noted as FP. (B) The phylogenetic tree of the panel of 16 HA subtypes found in avian reservoirs which are separated into five clades (color-coded) and can be segregated into two groups, Group-1 and Group-2.

We identified a conserved histidine at HA1 position 17 as a potential trigger residue for conformational change of Group-2 HAs. Histidine residues are of particular interest in this respect, as the imidazole side chains have a pKa of approximately 6.0 and therefore can become protonated in acidified endosomes. In Group-1 subtype HAs, HA1 position 17 is generally Tyr, so we attempted to identify an additional residue in these HAs that might play a similar role for triggering conformational changes during the entry process and focused our attention on HA2 residue 111. As seen in Figure 1, this highly conserved residue is similarly located in a region that becomes occupied by the fusion peptide following proteolytic activation of HA0, and it forms contacts with other highly conserved residues that are critical for fusion function such as HA2 Tyr 21. For the purpose of our studies, we cloned WT and mutant HAs representing a selection of Group-1 and Group-2 HAs for which we had suitable antisera for examining antigenic and biochemical properties of expressed HAs, as well as their functional phenotypes with respect to membrane fusion activity. The WT and mutant HAs utilized in the study are shown in Table 1, and include both human and avian representatives.

Table 1. Origin of HA subtype proteins

Origin	Subtypes	Strain name	17 mutation	111mutation
Avian	H1N1	A/duck/Alberta/35/1976	Y17H	H111A
Avian	H11N6	A/duck/England/1/1956	Y17H	H111A
Human	$H1N1_{PR8}$	A/Puerto Rico/8/1934	Y17H	H111A
Human	$H1N1_{CA}$	A/California/04/2009	Y17H	H111A
Human	$H2N2_{JAP}$	A/Japan/305/1957	Y17H	H111A
Human	$H5N1_{VN}$	A/Vietnam/1204/2004	Y17H	H111A
Avian	H3N8	A/duck/Ukraine/1/1963	H17Y	T111H
Avian	H10N7	A/chicken/Germany/N/1949	H17Y	T111H
Human	$H3N2_{Aichi}$	A/Aichi/2/1968	H17Y	T111H

3.2 Cell surface expression of HAs

Expression plasmids encoding the HAs shown in Table 1 were transfected into Vero cells and HA transport to the plasma membrane was examined using a cell surface biotinylation assay as shown in the gels in Figure 2 panel A. The bands representing surface-expressed HA were compared to total HA in the cell lysates of parallel assays, and the values of these are plotted as ratios with respect to corresponding WT HAs in panel B of Figure 2. It was determined that the HA1 17 mutants were expressed at levels ranging from 82% to 147% of wild-type indicating that the incorporation of histidine or tyrosine respectively did not significantly alter the expression profile of the HA. Similarly, for the HA2 111 mutants, surface expression relative to WT ranged from 44% to 192%, indicating that the HA2 position 111 HAs were also expressed at cell surfaces.

3.3 Cleavage activation of mutant HAs

In order to be primed for the acid-induced conformational changes that mediate membrane fusion, each monomer of the HA0 precursor structure must undergo proteolytic cleavage into the disulfide-linked subunits, HA1 and HA2. The HA of the H5 subtype A/Vietnam/1204/2004 contains a polybasic cleavage site that is recognized by cellular proteases and is expressed on the plasma membrane as cleaved HA; however, intracellular cleavage of the human $H5_{VN}$ HA H111A mutant was quite low, approximately 2-5 percent relative to WT, in agreement with a previous report [167]. The other HAs used in this study require addition of exogenous protease to activate

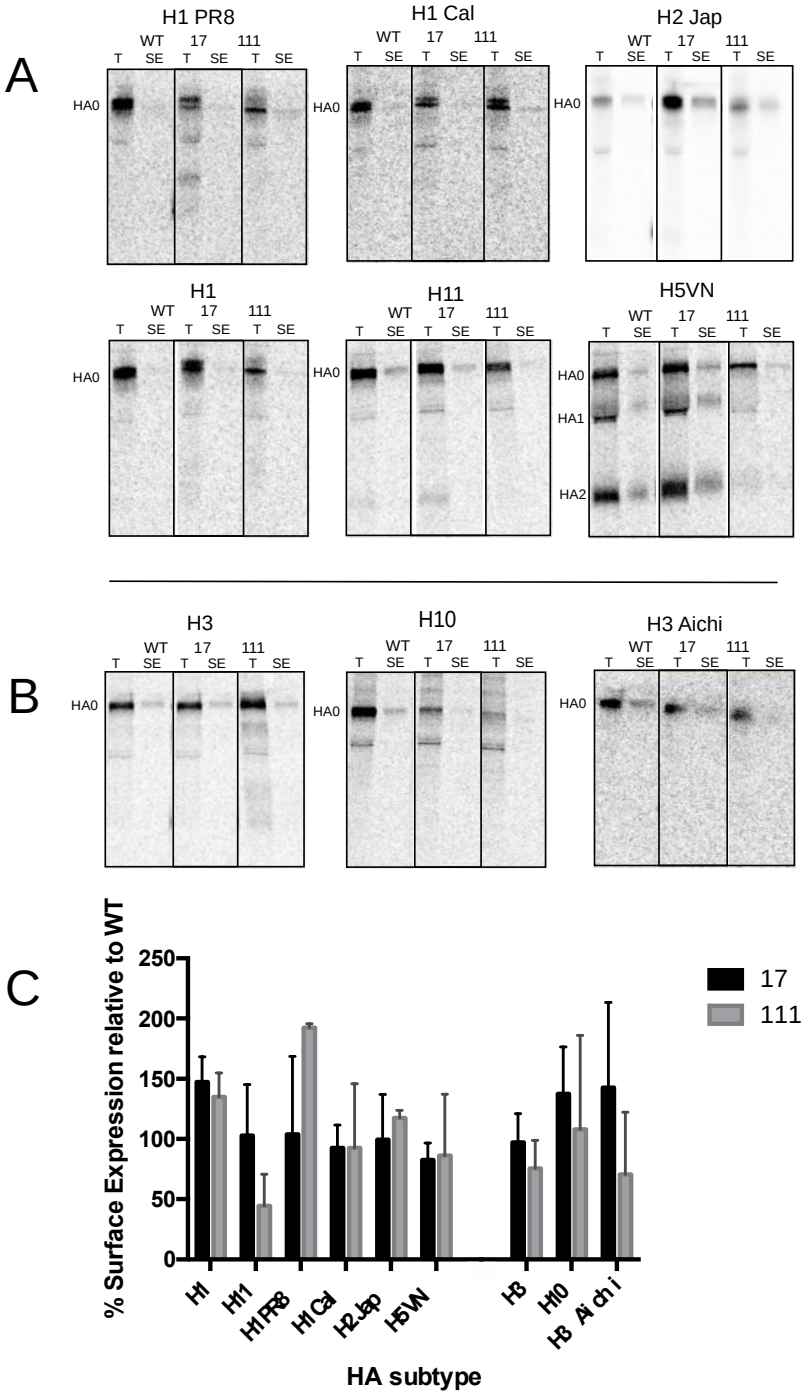

Figure 2. Surface expression of WT and mutant HAs by metabolic labelling and biotinylation of HA expressing cells. SDS-Page analysis, where lanes "T" represent total HA protein and "SE" represent surface expressed HA protein. (A) Gel images for human and avian subtypes corresponding to Group-1 are shown. (B) Gel images for human and avian subtypes corresponding to Group-2 are shown. (C) Quantitative representation of surface expressed mutants relative to the WT surface expression level. Error bars represent the standard deviation following three independent experiments.

fusion potential. To confirm that the mutant HAs can be proteolytically activated, HA proteins were expressed in the presence of [^{35}S]-methionine, cell monolayers were treated with exogenous trypsin or left untreated, and HA was precipitated with appropriate HA subtype-specific antibodies. Digestion products were then separated by SDS-PAGE under reducing conditions as shown in Figure 3, and bands corresponding to uncleaved HA0, and cleaved HA1 and HA2 subunits, were quantified by phosphoimager analysis. A comparison of mutant HA cleavage efficiency relative to the corresponding WT HA is shown in Figure 3 panel A, and reveals that for the HA1 17 mutations, percent cleavage compared to wild-type ranged from 70% to 143%, indicating that mutations at this residue did not significantly impact the capacity of mutant HAs to become proteolytically activated. For the HA2 111 mutations, most HAs were also cleaved with reasonable efficiency, ranging from 43% to 132% The results demonstrate that the mutant HAs can be activated for membrane fusion potential, and the cleavage into HA1 and HA2 by exogenous trypsin shows that they are transported to the plasma membrane, confirming the results of the surface biotinylation experiments described above.

3.4 Fusion activity and pH of fusion activation for mutant HAs

To examine the significance of the conserved residues at HA1 17 and HA2 111 for fusion activity, and the pH at which such activity is triggered, two assays were

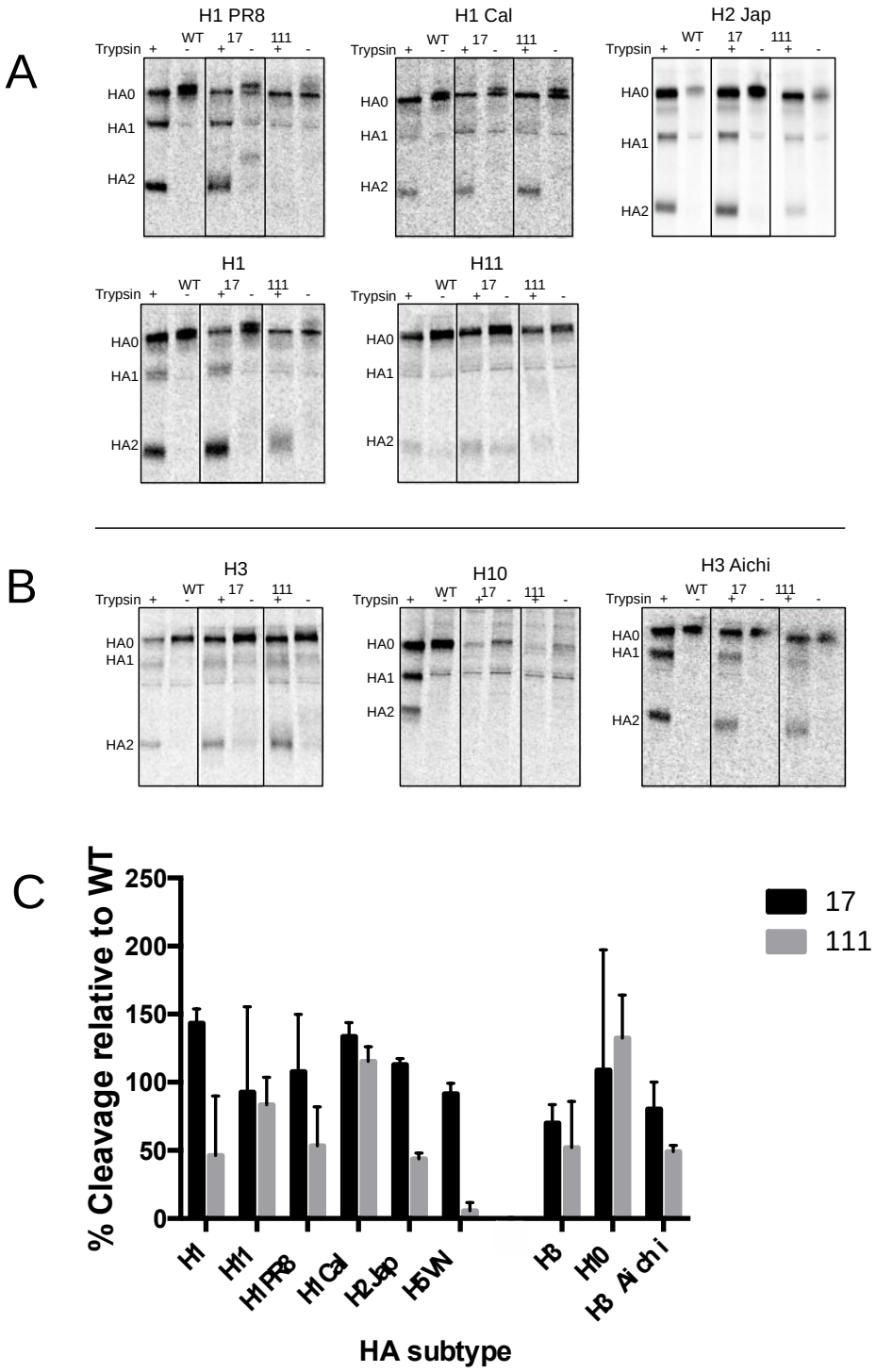

Figure 3. Proteolytic cleavage potential of WT and mutant HAs. Radiolabeled HA-expressing cells were treated with or without trypsin as indicated by + and – and lysates were immunoprecipitated with an HA-specific antibody and resolved using SDS-PAGE. (A) Gel images for both human and avian Group-1 HA subtypes. (B) Gel images for both human and avian Group-2 HA subtypes. (C) Quantitation of the trypsin cleavage of mutant HA subtypes relative to the WT HA.

employed. The first, a qualitative syncytia assay, allows for the visualization of fusion events resulting in the formation of multi-nucleated syncytia by HA-expressing cell monolayers following incubation at reduced pH. For this assay, HA-expressing cells are treated with trypsin to activate fusion potential, then incubated in acidic buffer over a range of pH values differing by increments of 0.1 units. The results of this assay are represented in Figure 4 where images of the monolayer at the pH of fusion, visualized by the highest pH value with observable syncytia, are shown next to images of the monolayers under buffered conditions at 0.1 pH units higher. If little or no syncytia were observed within the pH range predicted based on the pH of fusion for the WT HA, the monolayers were incubated at pH 4.4, the most acidic pH examined. For the three Group-2 HAs that were tested, the HA1 H17Y mutants displayed an acid-stable phenotype with fusion activity triggered at reduced pH relative to WT, and the HA2 H111A mutant HAs induced fusion at elevated pH relative to WT HA. These results are in agreement with previous reports on Group-2 HAs with substitutions at these positions [161]. For the Group-1 HAs, the HA1 Y17H mutations had destabilizing effects and an elevated fusion phenotype, but no fusion was detected at any pH for all HA2 H111A mutant proteins.

To corroborate these results, a quantitative luciferase-based reporter assay was carried out, which involves the transfection of the HA containing plasmid along with a plasmid containing the gene for luciferase under the control of a T7 promoter. Vero cells are transfected with these plasmids and treated with trypsin to activate surface expressed HAs. BSR-T7 cells that express the T7 polymerase are overlaid on the transfected Vero monolayer. The mixed cell populations are exposed to a low pH pulse,

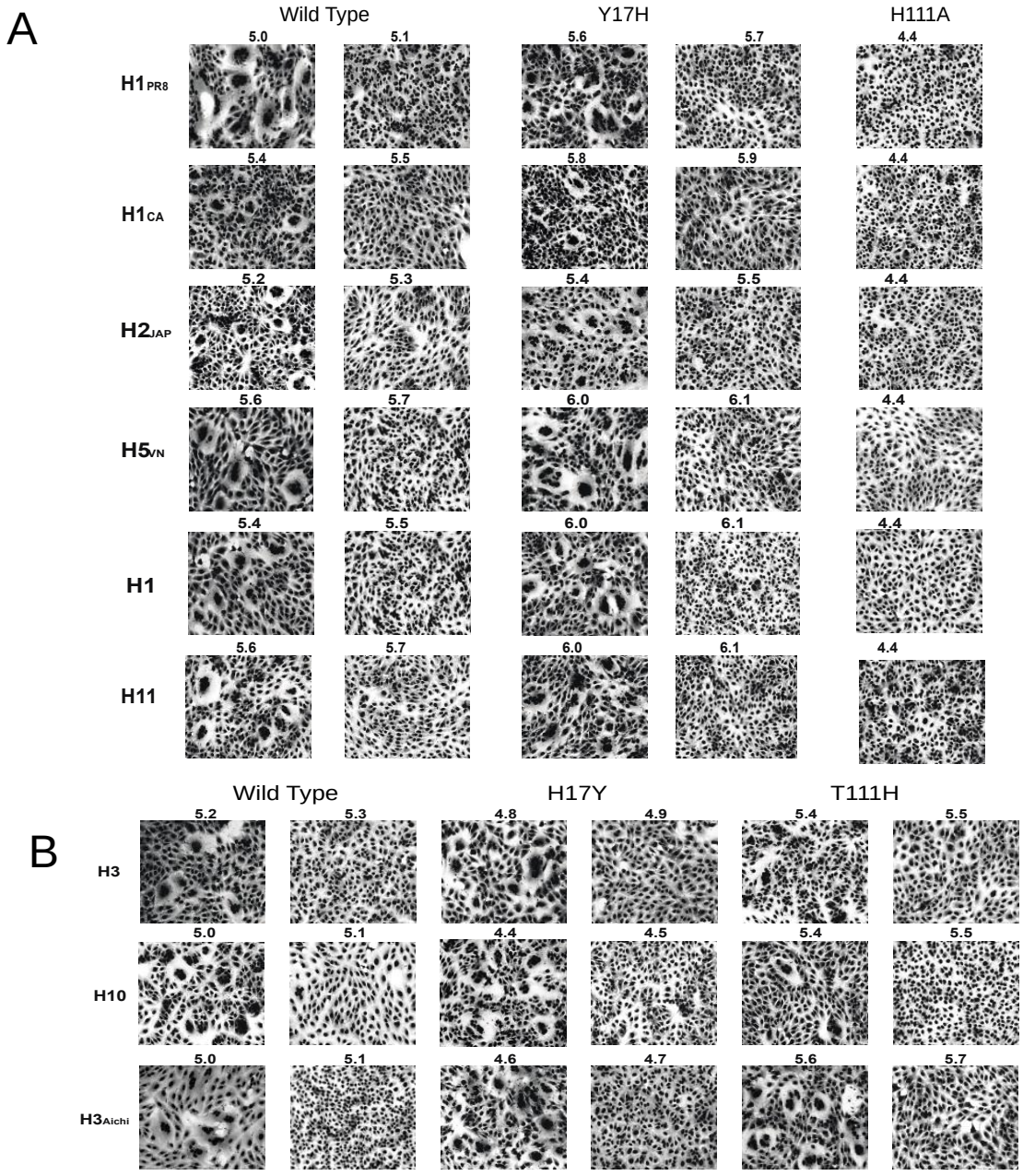

Figure 4. The pH of fusion as detected by the formation of syncytia. Photomicrographs of syncytia formation by avian and human Group-1 (A) and Group-2 (B) HA subtypes. BHK cells expressing HA were treated with trypsin and pH adjusted in 0.1 pH unit increments. The pH of fusion is shown as the highest pH at which syncytia were observed adjacent to cell monolayers the next increment lower, where fusion was significantly reduced by comparison.

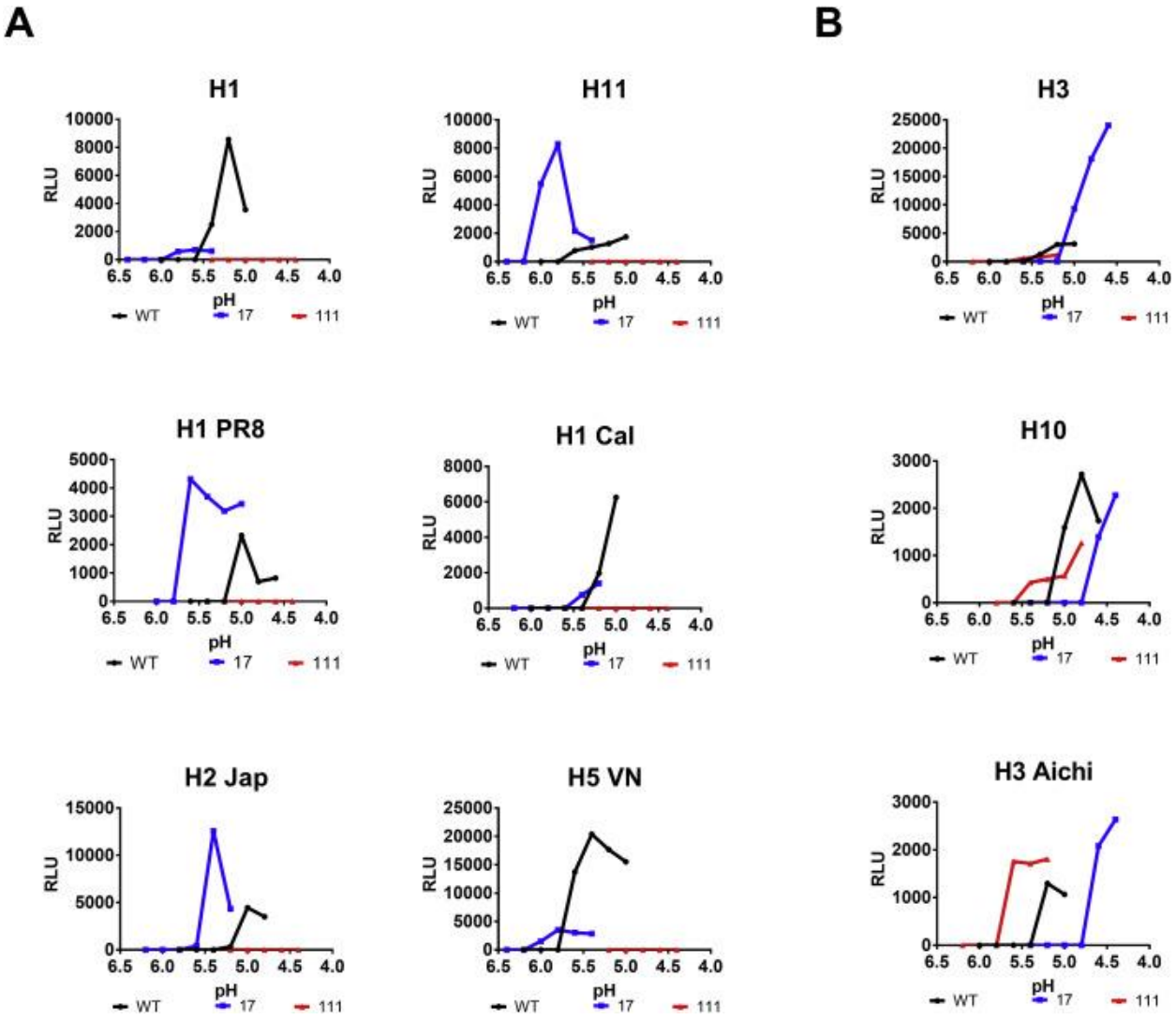

Figure 5. The pH of fusion as determined by the quantitative luciferase-based reporter assay. (A) Group-1 HA human and avian subtypes. Notably in this group, no fusion was detected of the H111A mutants. (B) Group-2 HA human and avian subtypes.

and functional HAs expressed on the Vero cells mediate fusion to the BSR-T7 cells, allowing for the transfer of the T7 polymerase and the expression of the luciferase reporter. The results of this assay for WT and mutant HAs are shown in Figure 5 and reflect those obtained with the syncytia assays both with respect to capacity of mutant HAs to mediate fusion, and the pH of fusion activity relative to corresponding WT HA.

Notably, no fusion was detected for any of the Group-1 HA2 H111A mutant HAs using either assay.

3.5 Conformational change of A/Japan/305/57 HAs

For the WT and HA2 H111A mutant HAs, it was possible to address the capacity to undergo acid-induced conformational changes with the conformation-specific monoclonal antibody 2/9, which reacts preferentially with the low pH structure [178, 180]. We used this monoclonal antibody, as well as a rabbit polyclonal antibody, to examine reactivity of HA-expressing cells by ELISA following treatment at pH 7.0 or 4.4. As shown in Figure 6, reactivity with the polyclonal serum showed that the WT HA expressed at higher levels relative to mutant, consistent with our surface biotinylation results, and also showed that antibody reactivity remained relatively unchanged following reduction in pH. The results with monoclonal antibody 2/9 show that reactivity to WT Japan HA is significantly greater following incubation at pH 4.4 compared to neutral pH, as expected for a fusion-active HA that had changed conformation as a result of acidification. However, no differences in reactivity with 2/9 were detected following acidification for the HA2 H111A mutant HA suggesting that it did not undergo the structural transitions required for membrane fusion activity.

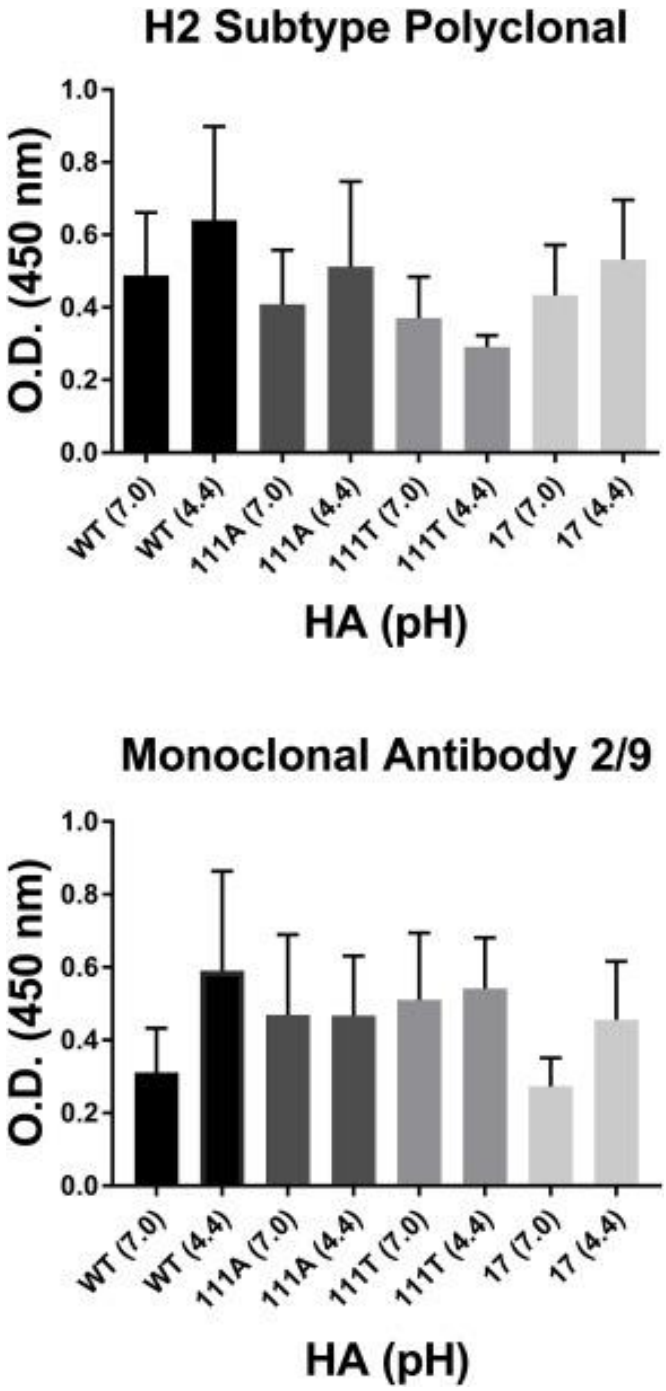

Figure 6. Conformational change of A/Japan/305/57 HAs. Enzyme-linked immunosorbent assay was performed as follows: Expressed HAs were trypsin treated followed by incubation at neutral pH (7.0) or low pH (4.4). (A) HA reactivity using H2 subtype polyclonal antibody. (B) HA reactivity using monoclonal antibody 2/9.

Table 2. Fusion pH of wild-type and mutant HAs

pH and △pH values for wild-type and mutant HAs

	Wild type		17 mutation				111 mutation			
	Luciferase	Syncytia	Luciferase		Syncytia		Luciferase		Syncytia	
Subtype			pH	△pH	pH	△pH	pH	△pH	pH	△pH
H1N1	5.4	5.4	5.8	0.4	6.0	0.6	n.o.*	–	n.o.	–
H11N6	5.6	5.6	6.0	0.4	6.0	0.4	n.o.	–	n.o.	–
H1N1$_{PR8}$	5.0	5.0	5.6	0.6	5.6	0.6	n.o.	–	n.o.	–
H1N1$_{CA}$	5.2	5.4	5.4	0.2	5.8	0.4	n.o.	–	n.o.	–
H2N2$_{JAP}$	5.0	5.2	5.4	0.4	5.4	0.2	n.o.	–	n.o.	–
H5N1$_{VN}$	5.6	5.6	6.0	0.4	6.0	0.4	n.o.	–	n.o.	–
H3N8	5.4	5.2	5.0	-0.4	4.8	-0.4	5.6	0.2	5.4	0.2
H10N7	5.0	5.0	4.6	-0.4	4.4	-0.6	5.4	0.4	5.4	0.4
H3N2$_{Aichi}$	5.0	5.0	4.6	-0.4	4.6	-0.4	5.6	0.6	5.6	0.6

n.o.: no HA fusion was observed at or above pH 4.4

4. Discussion

Several lines of evidence suggest that the initial triggers for the acid-induced structural rearrangements of HA that drive the membrane fusion process originate in the region of the fusion peptide. A comparison of the HA0 precursor structure [145] and that of the cleaved neutral pH HA [114] shows that only the residues of the HA0 cleavage loop relocate as a result of proteolytic activation (residues 323-328 of HA1 and 1-12 of HA2). However, uncleaved HA0 is relatively unresponsive to acidification, whereas the metastable cleaved HA can be triggered to undergo the well documented irreversible conformational changes that lead to fusion [206]. The major consequence of cleavage activation is that HA2 residues 1-12, the N-terminal domain of the fusion peptide, relocate into the trimer interior and bury a number of ionizable residues that line a cavity in the HA0 structure (Figure 7, panel A). The insertion of the HA2 N-terminus into the trimer interior primes the HA for fusion activity, and mutation of any residue within the first 10 positions of HA2, as well as several of the residues in the cavity that are buried, are known to destabilize the cleaved neutral pH HA [159, 170, 212-214]. In

studies using double mutants, the pH phenotype conferred by mutations in the fusion peptide region display dominance over changes more distal in the HA structure [173].

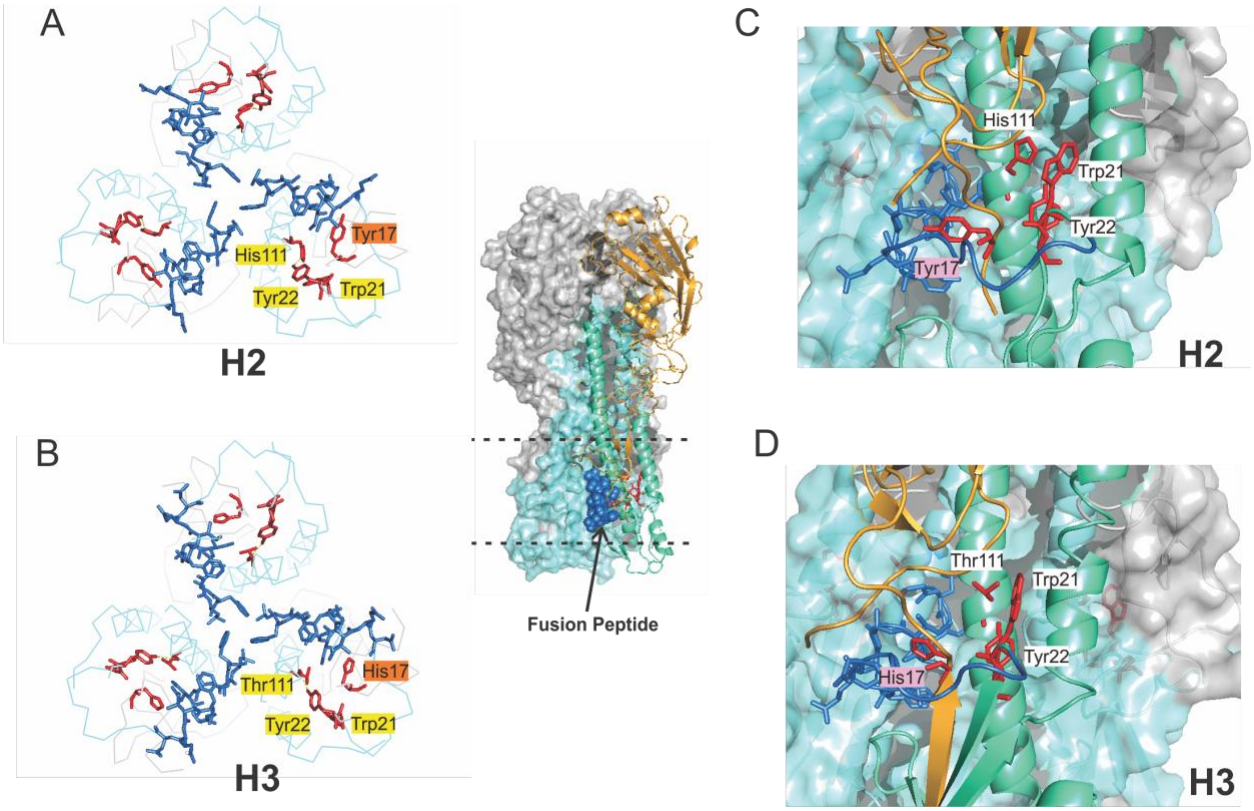

Figure 7. Structural depiction of HA2 position 111 region comparing Group-1 and Group-2 HAs, represented by H2 and H3 subtypes respectively. A full trimer viewed from the side is shown in the middle to indicate the region represented in the panels (area between hatched lines). Panels A and B represent views down the 3-fold axis of symmetry (top view) for H2 and H3 subtype HAs, and panels C and D show magnified views of these structures from the same orientation represented in the HA trimer at the center of the figure. Fusion peptide residues are shown in navy and group-specific residues HA1 17 and HA2 111, as well as highly conserved HA2 residues Trp21 and Tyr22, are shown in red.

Mutations in the fusion peptide region that elevate the pH of fusion have also been observed to confer resistance to a neutralizing monoclonal antibody that binds at monomer interfaces of the membrane distal head domains [216], and studies on the kinetics of acid-induced refolding using a panel of conformation-specific anti-peptide

monoclonal antibodies demonstrated that reactivity in the stem region preceded reactivity in the membrane-distal domains following acidification [174]. Also, analysis of the fusion kinetics of single virions using mutant viruses with changes in the fusion peptide, and/or HA2 residue 112 that lines the cavity, determined that withdrawal of the fusion peptide constitutes the rate-limiting step in the fusion process [175]. Studies using hydrogen-deuterium exchange and mass spectrometry to analyze the dynamics of acid-induced structural rearrangements lead to a similar conclusion, that extrusion of the fusion peptide precedes other events in the refolding process [67].

The results reported here confirm and extend a number of previous studies demonstrating that the HA region where fusion peptide residues insert into the trimer interior upon cleavage activation of the HA0 precursor, can play a critical role in HA stability and the priming of membrane fusion activity. The results with Group-2 mutant HAs for avian H3 and H10 strains support our previous results and conclusions based on the human H3 HA from A/Aichi/2/68 [161]. Specifically, these results confirm that changes at HA2 position 111 destabilize the Group-2 HAs resulting in a higher pH of fusion, whereas the introduction of Tyr for the conserved His at HA1 17 significantly stabilizes the structure, decreasing the pH of fusion by 0.4 or more in each case. These results support a role for HA1 His 17 as a potential trigger residue in the initiation of acid-induced conformational changes. When precursor HA0 is activated for fusion potential by proteolysis, the relocation of the newly-formed fusion peptide buries HA2 His 17, which forms hydrogen bonds to fusion peptide residues 6 and 10 via a water molecule [114, 145, 146], and the stabilizing phenotype conferred by a tyrosine substitution may be due in part to the longer side chain allowing formation of direct

hydrogen bonds with residues 10 and 12 of the fusion peptide. Of the conserved residues that are buried in Group-2 HAs, HA1 His 17 is a strong candidate to change protonation state during the pH transitions that would be encountered in the endosome. For the Group-1 HAs, the substitutions for Tyr at HA1 position 17 were all destabilizing, resulting in a higher pH of membrane fusion activity. Again, these may be due to the loss of hydrogen bonds formed between the Tyr and fusion peptide residues in the WT HA neutral pH structure. For the His to Ala substitution mutants at HA2 position 111, no fusion was detected at any pH examined. This suggests that the removal of the ionizable side chain of the His 111 residue in WT Group-1 HAs may render the structure unresponsive to acidification and suggests a potential role for the His 111 residues in initiating the conformational changes that mediate fusion activity. For the HA of A/Japan/305/57 virus, the conformation-specific monoclonal antibody 2/9 preferentially recognizes the low pH structure, and we demonstrated that WT Japan HA becomes significantly more reactive for MAb 2/9 upon acidification, whereas binding to mutant HA with Ala at HA2 position 111 showed no changes in reactivity following reduction in pH. The results indicate that the mutants were not undergo conformational changes and explain the loss of functional activity observed in the two membrane fusion assays.

For Group-1 HAs, the residue HA2 His 111 is buried following relocation of the fusion peptide upon activating cleavage. In the cleaved neutral pH structure, it resides in close proximity to completely conserved HA2 residues Trp21 and Tyr 22 and forms hydrogen bonds with the hydroxyl group on the side chain of the latter. A comparison of Group-1 HA structures and those of Group-2 HAs, which contain Thr at position 111, show that the residue at this position dictates the orientation of the indole side chain of

Trp 21, and structural features in this area might have critical implications for initiation of fusion. Both Trp 21 and Tyr 22 are conserved in all subtypes, and the structures of several HA-antibody complexes reveal that Trp 21 resides in the footprint of several broadly-reactive antibodies that can inhibit acid-induced conformational changes [183, 184, 201-203, 217-219]. Even though His 111 is not directly contacted by such antibodies, a His to Thr recombinant protein lost reactivity to one such antibody, C179 [184], and laboratory passage of an H5 subtype virus multiple times in the presence of another, CR6261, selected for a His to Leu substitution at position 111 [201]. In each case, the functional efficiency of the HA was not assessed, but we have observed that viruses with very poor fusion activity can be rescued by reverse genetics [198, 212, 213], including examples of mutants that were well documented for a hemifusion phenotype [220]. In these examples the virus titers in cell culture were orders of magnitude lower than WT, and following passage in the absence of any selective pressure, they reverted to either WT sequence or alternative amino acids. The threshold level of fusion activity required for virus fitness in natural environments is not known, but HA2 His 111 is conserved in all Group-1 HA subtypes.

Protonation of the His 111 side chain during endosomal acidification could potentially destabilize the structure in this region and help initiate the cascade of conformational changes that lead to membrane fusion. As discussed above, HA2 residues 106 and 111 are conserved in group-specific fashion, and the residues 106 through 112 are critical for function in that they undergo the helix-to-loop transition that results in formation of the helical bundles that draw viral and endosomal membranes into proximity to one another during fusion. Therefore, it is notable that mutagenesis

and X-ray crystallography studies on an H2 subtype HA with an Arg-to-His substitution at HA2 position 106 also reveal an acid-stable fusion phenotype [171]. Their structural data show that the side chain of the His 106 mutant HA rotates as acidification proceeds and structural changes translate to other regions of the HA in an intermediate “relaxed state”. They suggest that “a precise balance of charged residues” may help ensure that the HA structural transitions occur at optimal pH for endosomal fusion, and this would be consistent with our results with HA2 His 111 mutants. It could also be the case that HA2 His 111 is critical for ensuring that the structure in this region of the metastable pre-fusion HA is optimal for responding to acidification. A notable difference between Group-1 and Group-2 HAs is that the residue at HA2 111 influences H-bonds to Tyr22 and His 38 (in Group-1), and the orientation of the indole ring of Trp21 [146]; Figure 7, panels C and D). As noted above, mutation of position 111 can result in a loss of reactivity with fusion inhibiting antibodies that have Trp21 in the footprint. Recently, van Dongen et al., reported that an orally active antiviral compound based on one such antibody was effective against challenge by group-1 viruses of the H1 and H5 subtypes, and like the antibodies, the footprint of the drug includes residues Trp21 and His 38 [204]. A major challenge for the continued development of antiviral compounds such as this, or broadly effective “universal” vaccines will be the issues of drug resistance and escape from immune recognition, and the ongoing identification of residues, such as HA2 His 111, that are required for optimal function and fitness for a range of influenza subtypes will be critical.

Taken together with our current results, it would appear that the triggering of structural changes initiates at residues proximal to the fusion peptide such as HA2 106

and 111, and that these initiating events are then transmitted to the more distal domains prior to the “unclamping” of the membrane-distal head domains during the fusion process. Overall, if specific highly conserved residues such as HA2 His 111 in the fusion peptide region prove to be immutable for functionality, they may represent an “Achilles heel” target for fusion inhibitors that are resilient to drug resistance.

Acknowledgements

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Conclusion, Discussion, and Future Directions

For enveloped viruses, fusion is required between the viral membrane and cellular target membranes to release the viral genome within host cells and initiate viral replication. Enveloped viruses using fusion proteins include HIV, Ebola, SARS CoV, and influenza viruses, all of which cause infectious diseases of public health concern. Not all viral fusion proteins operate in the same way and can be classified into different classes of fusion proteins. The influenza hemagglutinin is a prototype Class I membrane fusion protein. Class I fusion proteins are defined by the presence of a fusion peptide that becomes the N-terminus of the fusogenic subunit itself following cleavage by an activating protease. These proteins undergo structural rearrangement to transition into a stable helical hairpin structure to mediate fusion. As well characterized and studied as the HA protein is, questions still remain regarding the process of fusion. A more thorough and detailed understanding of how this dynamic process occurs can lead to increased understanding of fusion proteins as well as strategies for the development of therapeutics to prevent disease.

Variations within the HA subtypes and strains lead to a different optimal pH of fusion for each virus, though the mechanism of structural rearrangement is well conserved within all the HA subtypes. Analysis of the residues that are conserved throughout the subtypes and groups can help focus our understanding of the function of these amino acid interactions and the overall mechanism of fusion. Previous work had identified, within group-2 subtypes, that His 17 in the HA1 subunit creates interactions within the fusion peptide and other residues and was shown to be an important trigger residue in the process of fusion. The pK_a of histidine is approximately 6.0 which allows

the histidine residues to be protonated and charged within the context of the low pH environment of an endosome. Histidine residues are often found within Class I and Class II viral fusion proteins and frequently are residues that are implicated in the structural changes which allow fusion to occur. Taking into context highly conserved residues found in the fusion peptide pocket as well as the importance of histidine residues found in viral fusion proteins and the ability for protonation in acidic environments, we narrowed our mutational interest within group-1 HA subtypes to a highly conserved histidine residue found in the HA2 subunit at position 111.

Initial experiments regarding the 111 residue of HA2 in the Group-1 subtypes used an alanine substitution at this site. Although this mutation allows us to explore the effect of removing a specific amino acid, in this case histidine, substitution with alanine may not allow us to understand if the His itself is required for function or if other amino acids could be incorporated at this site and maintain a fusogenic phenotype. Our work included additional site-directed mutagenesis of the 111 residue in the H2 subtype with other amino acids in place of the wild-type histidine and we showed that these mutant HAs are unable to mediate fusion. Use of pH conformation-specific antibodies directed against the H2 Japan subtype allows us to determine if a conformational change occurs following low pH exposure. Interestingly, we saw a clear indication that the leucine substitution at 111 may be resulting in a misfolded protein, evidenced by a smaller molecular weight protein band than the wild-type and other mutants (unpublished data). Additionally, conformation specific antibodies showed that the other mutants, Thr, Gln, and Ala, were not undergoing a structural change between neutral and low pH. We hypothesize that the structure of these HAs may be “locked” into a specific

conformation. In light of these results, the question remains if any amino acid could be substituted in place of the His residue of HA2 at position 111 within Group-1 subtypes which could result in a phenotype showing fusion.

The impact of this research becomes important when we evaluate the challenges influenza poses with respect to our current antiviral and vaccination strategies. Current antiviral strategies against influenza infection fall into two categories: M2 inhibitors and NA inhibitors. The two M2 ion channel inhibitors, amantadine and rimantadine, were originally used to effectively treat infection, however as ongoing use of these antiviral compounds occurred, resistance to these inhibitors spread [221-224]. These antivirals may be accessible for use against a novel influenza strain that has not commonly circulated in human populations, but resistance to these inhibitors may occur quickly. The other current antiviral strategy is to use neuraminidase inhibitors which prevent viral release from host cells, limiting infection. Neuraminidase inhibitors include zanamivir and oseltamivir which can broadly treat influenza A and B infections. Although resistance to these inhibitors can arise, the rates of resistance are lower for oseltamivir and even more rare for zanamivir than for resistance to M2 inhibitors. As a result, these inhibitors still being used for treatment against seasonal and pandemic viruses when necessary [225-228]. The logistics of effective treatment include the administration of these inhibitors within a timely fashion following infection, and as such, the use of inhibitors is not always an effective strategy for controlling the overall spread of influenza infection during seasonal epidemics or during pandemics, however, they can be utilized to help mitigate the severity of infection in certain situations.

A more appropriate measure for curtailing influenza infection and transmission is through the use of seasonal influenza vaccines. Current vaccination strategy largely relies on either trivalent (containing H1 and H3 IAV and either the Yamagata-like or Victoria-like IBV) or quadrivalent (containing H1 and H3 IAV and both lineages of IBV) vaccines directed at the HA [229-231]. Due to the high mutational rate and ongoing antigenic drift of the HA protein, the annual influenza vaccine must be continually evaluated and updated to attempt to elicit the best protection from the circulating and predominant HA strains which will occur in an upcoming season[232]. Regardless of natural infection or vaccination, the majority of the antibodies generated against influenza are antibodies which recognize the highly variable globular head domain of HA1, which serves as the most antigenically accessible region of the HA [233]. Constant antibody pressure on the immunodominant HA globular head leads to ongoing antigenic drift allowing the influenza virus to escape immune pressure. Due to the need for updating vaccines against seasonal strains, and the potential for pandemic viruses to emerge with novel subtypes, recent efforts have focused on development of universal flu vaccines to target more conserved epitopes, such as the HA stalk [234, 235] with the intention of generating more broadly neutralizing antibodies against the HA.

Broadly neutralizing antibodies (bnAbs) are antibodies capable of neutralizing more than one strain of the influenza virus, including some subtypes within a group, all subtypes within a group, or even subtypes in different groups [183, 217, 236, 237]. In the case of influenza HA bnAbs, this can occur through blocking the binding to the host cell by obscuring the RBS on HA or by binding to the conserved stem region often cross-linking the trimers, which might function to block the low pH conformational

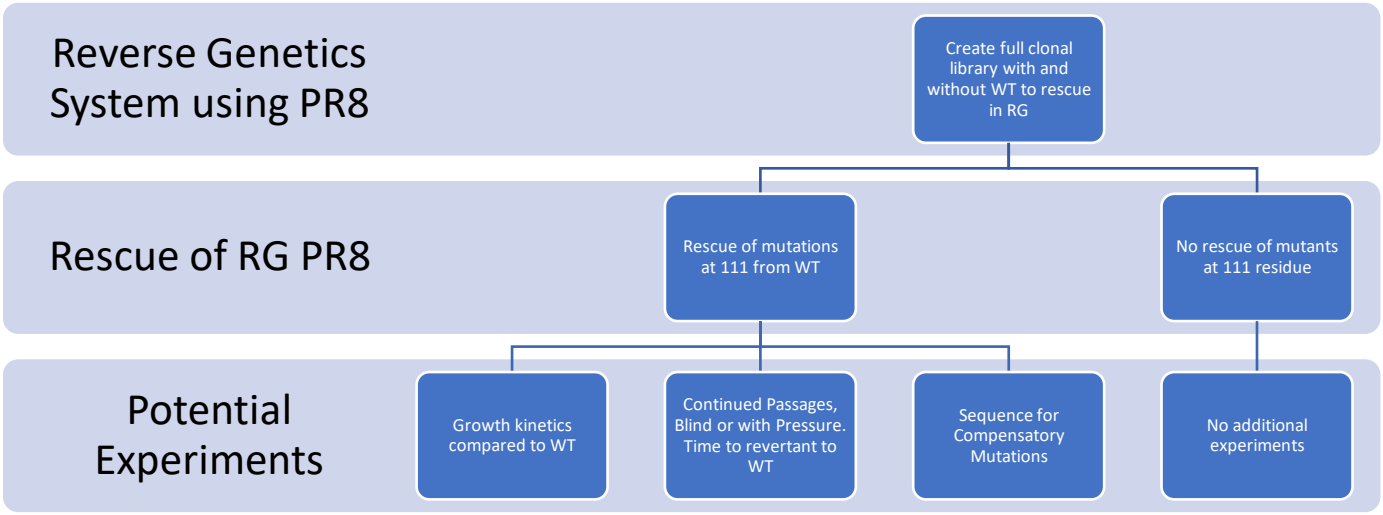
change required for fusion. The CR2621 antibody is able to neutralize multiple subtypes within group-1, including the H1, H2, and H5 subtypes, and does so through binding near a helical domain which blocks conformational change of HA[183]. Notably one of the first isolated broadly neutralizing antibodies, C179, was determined to bind to specific residues in HA1 (318-322) and HA2 (47-58), conserved among the H1 and H2 subtypes, leading to the neutralization of these viruses by inhibition of fusion[238]. Another antibody, F10, which can neutralize all group-1 subtypes was found to do so by inserting into the fusion peptide pocket of the subtypes, which due to the high conservation of the residues, allowed for broad reactivity [203]. Many of the bnAbs which target the stem region do so by binding hydrophobic or aromatic residues inside the hydrophobic groove of the stem region[203]. Escape mutants from bnAbs such as C179[238], CR8020[239], CR6261[201], and CR8043[240] have been observed, though with some impacts to the viral fitness such as reduced replication. Understanding the broadly neutralizing antibody binding and neutralization has also led to development of small molecule inhibitors that can prevent fusion by targeting epitopes of the bnAbs, as shown through the development of the small molecule inhibitor JNJ4796 which stabilizes the HA, blocking fusion, by targeting epitopes important in CR6261 binding [204]. Thus, as bnAb eliciting strategies are being explored for treatment and prevention of Influenza and for pandemic preparedness, it is important to understand the compensatory mutations that might arise should the conserved residues come under intense immune selection.

In our studies presented here, we may have identified a residue that is critical to triggering the structural rearrangements of HA leading to fusion. Since this residue is

conserved among group-1 subtypes, it becomes an attractive target for the generation of broadly neutralizing antibodies. However, such immune pressure has not been specifically applied and the “immutable” nature of His 111 has not truly been tested. Future work will examine the functionality of HA with mutations of every amino acid incorporated at 111 and possibly the additional mutations in surrounding areas that must also occur to ensure that HA is able to mediate fusion.

To address this line of work, we initiated reverse genetics studies using a set of plasmids containing the 8 gene segments of A/PR8/34 (H1N1) on a pDP backbone obtained from the Lowen Laboratory at Emory University. The plasmid containing the HA segment of PR8 was utilized to make a mutant library substituting 19 amino acids at HA2 111 in place of the wild-type His residue at that site. The mutant library was constructed with codon optimization of the mutants to be best suited for propagation in mammalian expression system, utilizing codons more frequently seen in mammalian cell lines, and generated by GenScript (Piscataway, NJ). Virus rescue experiments were carried out using standard procedures by transfection of plasmids in 293T cells and virus amplification in MDCK cells. Supernatants displayed positive cytopathic effects (CPE) and fusion of cells during rescue, similar to the wild-type positive control and not seen in negative controls, indicating that viruses were rescued. Determination of the rescued virus sequences are in progress at this time. Sequence analysis will reveal if observations of CPE and fusion were due to an immediate reversion to wild-type or if the rescued virus contains a sequence with a mutation at the 111 residue which is at least temporarily able to support a rescued virus without further passaging. With the rescue of these viruses, the flow of additional work is outlined

below and includes the experiments and potential future directions, see **Figure 1**.



If an established mutation at 111 is present, it will be interesting to determine whether optimal fusion function and virus replication are maintained, or whether compensatory mutations at other residues are contributing to function.

Future experiments on any rescued viruses will initially use serial passage to determine if reversion to wild-type occurs. If the wild-type residue of His is so critical to the fusion function that even mutants with compensatory mutations are less fit, a reversion to wild-type would be expected. Since the residue is so highly conserved within the Group-1 subtypes, the hypothesis is that the virus would not benefit from deviation from this highly conserved residue. Thus, defects in viral growth and kinetics between the wild-type and any rescued mutant virus may show a defect in the mutated residues even if compensatory mutations within the HA arise.

Should mutant viruses with changes at HA2 111 be confirmed, additional experiments will be conducted to understand the impact of the mutation on viral fitness.

Mutant virus stocks will be prepared, titered, and sequenced to confirm that the mutation is maintained. Replication kinetics of wild-type and mutant viruses will then be compared for single cycle growth conditions at a multiplicity of infection (MOI) of 5, and multicycle growth conditions at MOI of .001. Any rescued viruses with or without compensatory mutations can be evaluated for changes to the pH of fusion.

To understand the capacity for different amino acids to occupy position 111, deep sequencing of the supernatant of virus rescues will be used determine the makeup of quasispecies, if any are present. This would also allow us to determine the amino acid which are more prevalent substitutions than others, and which mutations may be more likely to emerge.

Finally, I hypothesize that 111 mutant HAs may not be able to tolerate compensatory mutations with equal fitness to wild-type. The surrounding residues and the 111 residue itself is highly conserved, and the residue itself is hypothesized to trigger fusion. A mutation from the His 111 may require compensatory mutations to occur within the fusion peptide pocket to replace any loss of stabilizing and triggering effects if the His residue were to be substituted with a less ionizable residue. Additional mutations may require several amino acid changes outside of the 111 residue to still allow for a fit virus, and even so fitness may be abrogated with so many mutations from conserved residues. These results would allow for understanding of not only the trigger residue as it pertains to fitness but also of the role of other residues which may directly or indirectly interact with the 111 residue as it relates to fusion and the optimal pH of fusion. This information would allow for multiple targets in antiviral and vaccination strategies, as we can focus not only on the residue of most interest, the “trigger”

residue, but also the residues within the environment which may be directly related to the ability for successful substitutions.

The identification of a highly conserved residue within a relatively conserved region of the HA stalk domain main have implications for future strategies for universal vaccines and development of antiviral compounds. This is even more significant since we have clearly demonstrated the functional significance which is likely to form the basis for conservation of HA2 His 111.

Future Ideas and Strategies

Broad implications of understanding the Class I fusion protein have the potential not only to better strategize for treatment and preparedness for IAV subtypes but can also be used to illuminate the fusion process for other fusion proteins for viruses such as Ebola, HIV, SARS CoV as well as other influenza types including the IBV and ICV. IBV may have similar conservation of residues and targets since IBV includes the HA protein much like IAV. Unlike IAV and IBV, ICV has a hemagglutinin-esterase-fusion (HEF) protein which has approximately 12% sequence identity to the HA of IAV and IBV. Due to the HEF protein serving the same function as the HA of IAV and IBV, it must undergo similar fusion changes in the endosome but this process is not as well characterized. Understanding the process of fusion in ICV HEF may lead to important clues in understanding other distinct fusion proteins, as well as the level of changes which the HA protein may be able to tolerate and perform similar function. Understanding how markedly changes in the HA from IAV to IBV and ICV occur can be

used in understanding other viral fusion proteins by further extrapolating the comparison between IAV and IBV or ICV in conjunction with other distinct fusion processes in viruses with similar concern to public health. Understanding changes within influenza types may be especially important when evaluating the breadth of response to selective pressures of IAV HA which can tolerate changes but maintain the same functions necessary for the viral life cycle. The pathology of ICV is considerably more mild in humans than that of IAV and IBV and understanding if the HA to HEF differences relate to the overall pathology of the virus can help understand the fitness defects that may occur with great change to the HA protein when mutated.

While the HA is one of the most well-studied and characterized Class I fusion proteins, there are other similar fusion proteins in a multitude of pathogens which utilize a similar low pH fusion trigger and structural rearrangements. A detailed understanding of the influenza HA fusion process can point to interrogating other diseases operating under similar fusion mechanisms. Understanding some of the important residues, microenvironments of fusion peptides, and the cellular biology of host cells which play a role in infection can lead to a broader understanding of other fusion proteins. Fusion proteins of other viruses have conserved residues, specifically histidine residues interacting with other amino acids, creating “fusion trigger” hotspots that may useful to study further for similar dynamics of mutation and pressure via antiviral and vaccination strategies [241-243].

Lastly, the environment of the host, within the context of the host cells but also the external environment and route of transmission, is clearly important in influenza infection. Co-infections within the respiratory tract have the potential to change the

environment of the respiratory tract. Infection with another respiratory pathogen can increase the chance for secondary influenza infection and may play a role in the infection and pathogenesis of both the initial infection and subsequent influenza infection. Co-infection of viral or bacterial pathogens with influenzas occurs and can lead to more severe pathology in patients. *Pseudomonas aeruginosa* is a common bacterial pathogen which is often problematic for patients with cystic fibrosis (CF). Interestingly the pH of the respiratory tract within CF patients is disrupted from the pH found within the normal respiratory tract. The nasal cavity of CF patients is more acidic, with an average pH of 5.2 compared to 6.3 in healthy adults. This lower pH environment may be more resistant to influenza infection, if the pH is at or below that of HA fusion in the nasal passages [244, 245]. Similar changes in pH of the mucosa during bacterial infections may impact successful viral infection or co-infection with bacterial respiratory pathogens. Does bacterial infection and biofilm formation alter the pH of the respiratory tract and enhance the capacity for viral infection or persistence and disease pathology of either infection? A “whole system” approach examining HA stability and functionality in various pH environments, amongst co-infection or other disease states, and in the context of mucosal viscosity or the inflammatory response within the lung may help us better understand the overall stability and transmissibility of influenza. By identifying environments which decrease or challenge influenza infection, as well as understanding what may enhance the viral fitness of influenza, we will be better equipped for developing successful treatment and prevention strategies.

References

1. Hause, B.M., et al., *Characterization of a novel influenza virus in cattle and Swine: proposal for a new genus in the Orthomyxoviridae family.* MBio, 2014. **5**(2): p. e00031-14.
2. Taylor, R.M., *Studies on survival of influenza virus between epidemics and antigenic variants of the virus.* American journal of public health and the nation's health, 1949. **39**(2): p. 171-178.
3. *A revised system of nomenclature for influenza viruses.* Bulletin of the World Health Organization, 1971. **45**(1): p. 119-124.
4. *A revision of the system of nomenclature for influenza viruses: a WHO memorandum.* Bull World Health Organ, 1980. **58**(4): p. 585-91.
5. Tong, S., et al., *New world bats harbor diverse influenza A viruses.* PLoS Pathog, 2013. **9**(10): p. e1003657.
6. Nobusawa, E., et al., *Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses.* Virology, 1991. **182**(2): p. 475-85.
7. Air, G.M., *Sequence relationships among the hemagglutinin genes of 12 subtypes of influenza A virus.* Proc Natl Acad Sci U S A, 1981. **78**(12): p. 7639-43.
8. Rolfes, M.A., et al., *Annual estimates of the burden of seasonal influenza in the United States: A tool for strengthening influenza surveillance and preparedness.* Influenza and other respiratory viruses, 2018. **12**(1): p. 132-137.
9. Finkelman, B.S., et al., *Global patterns in seasonal activity of influenza A/H3N2, A/H1N1, and B from 1997 to 2005: viral coexistence and latitudinal gradients.* PloS one, 2007. **2**(12): p. e1296-e1296.
10. Rambaut, A., et al., *The genomic and epidemiological dynamics of human influenza A virus.* Nature, 2008. **453**(7195): p. 615-9.
11. Dave, K. and P.C. Lee, *Global Geographical and Temporal Patterns of Seasonal Influenza and Associated Climatic Factors.* Epidemiologic Reviews, 2019.
12. Lowen, A.C., et al., *Influenza virus transmission is dependent on relative humidity and temperature.* PLoS Pathog, 2007. **3**(10): p. 1470-6.
13. Bush, R.M., et al., *Predicting the Evolution of Human Influenza A.* Science, 1999. **286**(5446): p. 1921.
14. Park, W.H., et al., *STUDIES ON THE ETIOLOGY OF THE PANDEMIC OF 1918.* American journal of public health (New York, N.Y. : 1912), 1919. **9**(1): p. 45-49.
15. Murray, C.J.L., et al., *Estimation of potential global pandemic influenza mortality on the basis of vital registry data from the 1918–20 pandemic: a quantitative analysis.* The Lancet, 2006. **368**(9554): p. 2211-2218.
16. Schäffr, J.R., et al., *Origin of the Pandemic 1957 H2 Influenza A Virus and the Persistence of Its Possible Progenitors in the Avian Reservoir.* Virology, 1993. **194**(2): p. 781-788.
17. Cockburn, W.C., P.J. Delon, and W. Ferreira, *Origin and progress of the 1968-69 Hong Kong influenza epidemic.* Bulletin of the World Health Organization, 1969. **41**(3): p. 345-348.
18. Zarocostas, J., *World Health Organization declares A (H1N1) influenza pandemic.* BMJ, 2009. **338**: p. b2425.

19. Buonagurio, D.A., et al., *Evolution of human influenza A viruses over 50 years: rapid, uniform rate of change in NS gene.* Science, 1986. **232**(4753): p. 980-2.
20. Chen, R. and E.C. Holmes, *Avian Influenza Virus Exhibits Rapid Evolutionary Dynamics.* Molecular Biology and Evolution, 2006. **23**(12): p. 2336-2341.
21. Fitch, W.M., et al., *Long term trends in the evolution of H(3) HA1 human influenza type A.* Proceedings of the National Academy of Sciences, 1997. **94**(15): p. 7712.
22. Fourment, M., et al., *Evolutionary dynamics of the N1 neuraminidases of the main lineages of influenza A viruses.* Molecular Phylogenetics and Evolution, 2010. **56**(2): p. 526-535.
23. Gorman, O.T., et al., *Evolution of the nucleoprotein gene of influenza A virus.* J Virol, 1990. **64**(4): p. 1487-97.
24. Verhoeyen, M., et al., *Antigenic drift between the haemagglutinin of the Hong Kong influenza strains A/Aichi/2/68 and A/Victoria/3/75.* Nature, 1980. **286**(5775): p. 771-776.
25. Knossow, M. and J.J. Skehel, *Variation and infectivity neutralization in influenza.* Immunology, 2006. **119**(1): p. 1-7.
26. Ferguson, N.M., A.P. Galvani, and R.M. Bush, *Ecological and immunological determinants of influenza evolution.* Nature, 2003. **422**(6930): p. 428-433.
27. Kawaoka, Y., S. Krauss, and R.G. Webster, *Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics.* J Virol, 1989. **63**(11): p. 4603-8.
28. Laver, W.G., J.C. Downie, and R.G. Webster, *Studies on antigenic variation in influenza virus. Evidence for multiple antigenic determinants on the hemagglutinin subunits of A-Hong Kong-68 (H3 N2) virus and the A-England-72 strains.* Virology, 1974. **59**(1): p. 230-44.
29. *Emergence of a Novel Swine-Origin Influenza A (H1N1) Virus in Humans.* New England Journal of Medicine, 2009. **360**(25): p. 2605-2615.
30. Neumann, G., T. Noda, and Y. Kawaoka, *Emergence and pandemic potential of swine-origin H1N1 influenza virus.* Nature, 2009. **459**(7249): p. 931-9.
31. Hancock, K., et al., *Cross-Reactive Antibody Responses to the 2009 Pandemic H1N1 Influenza Virus.* N Engl J Med, 2009.
32. Krause, J.C., et al., *Naturally Occurring Human Monoclonal Antibodies Neutralize both 1918 and 2009 Pandemic Influenza A (H1N1) Viruses.* Journal of Virology, 2010. **84**(6): p. 3127.
33. Downie, J.C. and W.G. Laver, *Isolation of a type A influenza virus from an Australian pelagic bird.* Virology, 1973. **51**(2): p. 259-269.
34. Slemons, R.D., et al., *Type-A Influenza Viruses Isolated from Wild Free-Flying Ducks in California.* Avian Diseases, 1974. **18**(1): p. 119-124.
35. Webster, R.G., et al., *Intestinal influenza: replication and characterization of influenza viruses in ducks.* Virology, 1978. **84**(2): p. 268-78.
36. Ito, T., et al., *Recognition of N-glycolylneuraminic acid linked to galactose by the alpha2,3 linkage is associated with intestinal replication of influenza A virus in ducks.* J Virol, 2000. **74**(19): p. 9300-5.
37. Bröjer, C., et al., *Pathology of Natural Highly Pathogenic Avian Influenza H5N1 Infection in Wild Tufted Ducks (Aythya Fuligula).* Journal of Veterinary Diagnostic Investigation, 2009. **21**(5): p. 579-587.

38. *Experimental Exposure of Human Subjects to Viruses of Influenza.* The Journal of Immunology, 1946. **52**(2): p. 145.
39. Ito, T. and Y. Kawaoka, *Host-range barrier of influenza A viruses.* Vet Microbiol, 2000. **74**(1-2): p. 71-5.
40. Couceiro, J.N., J.C. Paulson, and L.G. Baum, *Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity.* Virus Res, 1993. **29**(2): p. 155-65.
41. Nelli, R.K., et al., *Comparative distribution of human and avian type sialic acid influenza receptors in the pig.* BMC Veterinary Research, 2010. **6**(1): p. 4.
42. Meier-Ewert, H., A.J. Gibbs, and N.J. Dimmock, *Studies on Antigenic Variations of the Haemagglutinin and Neuraminidase of Swine Influenza Virus Isolates.* Journal of General Virology, 1970. **6**(3): p. 409-419.
43. Ito, M., et al., *Genetic Analyses of an H3N8 Influenza Virus Isolate, Causative Strain of the Outbreak of Equine Influenza at the Kanazawa Racecourse in Japan in 2007.* Journal of Veterinary Medical Science, 2008. **70**(9): p. 899-906.
44. Gibson, C.A., et al., *Sequence analysis of the equine H7 influenza virus haemagglutinin gene.* Virus Research, 1992. **22**(2): p. 93-106.
45. Suzuki, Y., et al., *Sialic acid species as a determinant of the host range of influenza A viruses.* J Virol, 2000. **74**(24): p. 11825-31.
46. Gambaryan, A.S., et al., *Receptor-binding profiles of H7 subtype influenza viruses in different host species.* Journal of virology, 2012. **86**(8): p. 4370-4379.
47. Shinya, K., et al., *Avian flu: influenza virus receptors in the human airway.* Nature, 2006. **440**(7083): p. 435-6.
48. Matrosovich, M., et al., *Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals.* J Virol, 2000. **74**(18): p. 8502-12.
49. Lamb, R.A. and P.W. Choppin, *Identification of a second protein (M2) encoded by RNA segment 7 of influenza virus.* Virology, 1981. **112**(2): p. 729-737.
50. Lamb, R.A. and P.W. Choppin, *Segment 8 of the influenza virus genome is unique in coding for two polypeptides.* Proceedings of the National Academy of Sciences, 1979. **76**(10): p. 4908.
51. Wise, H.M., et al., *A Complicated Message: Identification of a Novel PB1-Related Protein Translated from Influenza A Virus Segment 2 mRNA.* Journal of Virology, 2009. **83**(16): p. 8021.
52. Jagger, B.W., et al., *An Overlapping Protein-Coding Region in Influenza A Virus Segment 3 Modulates the Host Response.* Science, 2012. **337**(6091): p. 199.
53. Connor, R.J., et al., *Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates.* Virology, 1994. **205**(1): p. 17-23.
54. Doms, R.W., A. Helenius, and J. White, *Membrane fusion activity of the influenza virus hemagglutinin. The low pH-induced conformational change.* Journal of Biological Chemistry, 1985. **260**(5): p. 2973-2981.
55. Skehel, J.J. and D.C. Wiley, *Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin.* Annu Rev Biochem, 2000. **69**: p. 531-69.

56. Wiley, D.C. and J.J. Skehel, *The structure and function of the hemagglutinin membrane glycoprotein of influenza virus.* Annu Rev Biochem, 1987. **56**: p. 365-94.
57. Palese, P., et al., *Characterization of temperature sensitive influenza virus mutants defective in neuraminidase.* Virology, 1974. **61**(2): p. 397-410.
58. Takeda, M., et al., *Influenza a virus M2 ion channel activity is essential for efficient replication in tissue culture.* J Virol, 2002. **76**(3): p. 1391-9.
59. Chizhmakov, I.V., et al., *Selective proton permeability and pH regulation of the influenza virus M2 channel expressed in mouse erythroleukaemia cells.* The Journal of Physiology, 1996. **494**(2): p. 329-336.
60. Zhirnov, O.P., *Solubilization of matrix protein M1/M from virions occurs at different pH for orthomyxo- and paramyxoviruses.* Virology, 1990. **176**(1): p. 274-279.
61. Gamblin, S.J. and J.J. Skehel, *Influenza hemagglutinin and neuraminidase membrane glycoproteins.* J Biol Chem, 2010. **285**(37): p. 28403-9.
62. Harris, A., et al., *Influenza virus pleiomorphy characterized by cryoelectron tomography.* Proc Natl Acad Sci U S A, 2006. **103**(50): p. 19123-7.
63. Varghese, J.N., W.G. Laver, and P.M. Colman, *Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 Å resolution.* Nature, 1983. **303**(5912): p. 35-40.
64. Sieczkarski, S.B. and G.R. Whittaker, *Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis.* J Virol, 2002. **76**(20): p. 10455-64.
65. Matlin, K.S., et al., *Infectious entry pathway of influenza virus in a canine kidney cell line.* J Cell Biol, 1981. **91**(3 Pt 1): p. 601-13.
66. Skehel, J.J., et al., *Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion.* Proc Natl Acad Sci U S A, 1982. **79**(4): p. 968-72.
67. Garcia, N.K., et al., *Dynamic changes during acid-induced activation of influenza hemagglutinin.* Structure (London, England : 1993), 2015. **23**(4): p. 665-676.
68. Qin, C., et al., *Real-time dissection of dynamic uncoating of individual influenza viruses.* Proceedings of the National Academy of Sciences, 2019. **116**(7): p. 2577.
69. Hsu, M.T., et al., *Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle.* Proceedings of the National Academy of Sciences, 1987. **84**(22): p. 8140.
70. Li, M.L., B.C. Ramirez, and R.M. Krug, *RNA-dependent activation of primer RNA production by influenza virus polymerase: different regions of the same protein subunit constitute the two required RNA-binding sites.* EMBO J, 1998. **17**(19): p. 5844-52.
71. Li, X. and P. Palese, *Characterization of the polyadenylation signal of influenza virus RNA.* Journal of virology, 1994. **68**(2): p. 1245-1249.
72. Robertson, J.S., M. Schubert, and R.A. Lazzarini, *Polyadenylation sites for influenza virus mRNA.* Journal of virology, 1981. **38**(1): p. 157-163.
73. Plotch, S.J., et al., *A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription.* Cell, 1981. **23**(3): p. 847-58.
74. Plotch, S.J., M. Bouloy, and R.M. Krug, *Transfer of 5'-terminal cap of globin mRNA to influenza viral complementary RNA during transcription in vitro.* Proceedings of

the National Academy of Sciences of the United States of America, 1979. **76**(4): p. 1618-1622.
75. Lamb, R.A. and C.-J. Lai, *Spliced and unspliced messenger RNAs synthesized from cloned influenza virus M DNA in an SV40 vector: Expression of the influenza virus membrane protein (M1).* Virology, 1982. **123**(2): p. 237-256.
76. Bouvier, N.M. and P. Palese, *The biology of influenza viruses.* Vaccine, 2008. **26 Suppl 4**(Suppl 4): p. D49-D53.
77. Hatada, E., et al., *Control of Influenza Virus Gene Expression: Quantitative Analysis of Each Viral RNA Species in Infected Cells.* The Journal of Biochemistry, 1989. **105**(4): p. 537-546.
78. Hay, A.J., et al., *Transcription of the influenza virus genome.* Virology, 1977. **83**(2): p. 337-55.
79. Martin, K. and A. Helenius, *Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import.* Cell, 1991. **67**(1): p. 117-30.
80. Huang, X., et al., *Effect of Influenza Virus Matrix Protein and Viral RNA on Ribonucleoprotein Formation and Nuclear Export.* Virology, 2001. **287**(2): p. 405-416.
81. Neumann, G., M.R. Castrucci, and Y. Kawaoka, *Nuclear import and export of influenza virus nucleoprotein.* J Virol, 1997. **71**(12): p. 9690-700.
82. Neumann, G., M.T. Hughes, and Y. Kawaoka, *Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1.* EMBO J, 2000. **19**(24): p. 6751-8.
83. Boulan, E.R. and D.D. Sabatini, *Asymmetric budding of viruses in epithelial monlayers: a model system for study of epithelial polarity.* Proceedings of the National Academy of Sciences, 1978. **75**(10): p. 5071.
84. Hughey, P.G., et al., *Expression of the influenza A virus M2 protein is restricted to apical surfaces of polarized epithelial cells.* J Virol, 1992. **66**(9): p. 5542-52.
85. Jones, L.V., et al., *Surface expression of influenza virus neuraminidase, an amino-terminally anchored viral membrane glycoprotein, in polarized epithelial cells.* Mol Cell Biol, 1985. **5**(9): p. 2181-9.
86. Roth, M.G., et al., *Influenza virus hemagglutinin expression is polarized in cells infected with recombinant SV40 viruses carrying cloned hemagglutinin DNA.* Cell, 1983. **33**(2): p. 435-43.
87. Enami, M. and K. Enami, *Influenza virus hemagglutinin and neuraminidase glycoproteins stimulate the membrane association of the matrix protein.* Journal of virology, 1996. **70**(10): p. 6653-6657.
88. Doms, R.W., et al., *Folding and Assembly of Viral Membrane Proteins.* Virology, 1993. **193**(2): p. 545-562.
89. Sugrue, R.J., R.B. Belshe, and A.J. Hay, *Palmitoylation of the influenza a virus M2 protein.* Virology, 1990. **179**(1): p. 51-56.
90. Veit, M., et al., *Site-specific mutagenesis identifies three cysteine residues in the cytoplasmic tail as acylation sites of influenza virus hemagglutinin.* J Virol, 1991. **65**(5): p. 2491-500.
91. Steinhauer, D.A., et al., *Amantadine selection of a mutant influenza virus containing an acid-stable hemagglutinin glycoprotein: evidence for virus-specific*

regulation of the pH of glycoprotein transport vesicles. Proc Natl Acad Sci U S A, 1991. **88**(24): p. 11525-9.
92. Stieneke-Grober, A., et al., *Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease.* EMBO J, 1992. **11**(7): p. 2407-14.
93. Klenk, H.D., et al., *Activation of influenza A viruses by trypsin treatment.* Virology, 1975. **68**(2): p. 426-39.
94. Barman, S., et al., *Role of transmembrane domain and cytoplasmic tail amino acid sequences of influenza a virus neuraminidase in raft association and virus budding.* J Virol, 2004. **78**(10): p. 5258-69.
95. Kundu, A., et al., *Transmembrane domain of influenza virus neuraminidase, a type II protein, possesses an apical sorting signal in polarized MDCK cells.* Journal of virology, 1996. **70**(9): p. 6508-6515.
96. Scheiffele, P., et al., *Influenza viruses select ordered lipid domains during budding from the plasma membrane.* J Biol Chem, 1999. **274**(4): p. 2038-44.
97. Takeda, M., et al., *Influenza virus hemagglutinin concentrates in lipid raft microdomains for efficient viral fusion.* Proc Natl Acad Sci U S A, 2003. **100**(25): p. 14610-7.
98. Ali, A., et al., *Influenza virus assembly: effect of influenza virus glycoproteins on the membrane association of M1 protein.* Journal of virology, 2000. **74**(18): p. 8709-8719.
99. Furuse, Y. and H. Oshitani, *Evolution of the influenza A virus untranslated regions.* Infection, Genetics and Evolution, 2011. **11**(5): p. 1150-1154.
100. Gog, J.R., et al., *Codon conservation in the influenza A virus genome defines RNA packaging signals.* Nucleic Acids Research, 2007. **35**(6): p. 1897-1907.
101. Noda, T., et al., *Architecture of ribonucleoprotein complexes in influenza A virus particles.* Nature, 2006. **439**(7075): p. 490-2.
102. Fujii, Y., et al., *Selective incorporation of influenza virus RNA segments into virions.* Proc Natl Acad Sci U S A, 2003. **100**(4): p. 2002-7.
103. Jacobs, N.T., et al., *Incomplete influenza A virus genomes occur frequently but are readily complemented during localized viral spread.* Nature communications, 2019. **10**(1): p. 3526-3526.
104. Fonville, J.M., et al., *Influenza Virus Reassortment Is Enhanced by Semi-infectious Particles but Can Be Suppressed by Defective Interfering Particles.* PLoS Pathog, 2015. **11**(10): p. e1005204.
105. White, M.C., J. Steel, and A.C. Lowen, *Heterologous Packaging Signals on Segment 4, but Not Segment 6 or Segment 8, Limit Influenza A Virus Reassortment.* Journal of virology, 2017. **91**(11): p. e00195-17.
106. White, M.C., et al., *H5N8 and H7N9 packaging signals constrain HA reassortment with a seasonal H3N2 influenza A virus.* Proceedings of the National Academy of Sciences of the United States of America, 2019. **116**(10): p. 4611-4618.
107. Bourmakina, S.V. and A. Garcia-Sastre, *Reverse genetics studies on the filamentous morphology of influenza A virus.* J Gen Virol, 2003. **84**(Pt 3): p. 517-27.
108. Elleman, C.J. and W.S. Barclay, *The M1 matrix protein controls the filamentous phenotype of influenza A virus.* Virology, 2004. **321**(1): p. 144-53.

109. Campbell, P.J., et al., *Residue 41 of the Eurasian avian-like swine influenza a virus matrix protein modulates virion filament length and efficiency of contact transmission.* J Virol, 2014. **88**(13): p. 7569-77.
110. Burleigh, L.M., et al., *Influenza a viruses with mutations in the m1 helix six domain display a wide variety of morphological phenotypes.* J Virol, 2005. **79**(2): p. 1262-70.
111. Liu, C., et al., *Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding.* J Virol, 1995. **69**(2): p. 1099-106.
112. Palese, P. and R.W. Compans, *Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): mechanism of action.* J Gen Virol, 1976. **33**(1): p. 159-63.
113. Wagner, R., M. Matrosovich, and H.D. Klenk, *Functional balance between haemagglutinin and neuraminidase in influenza virus infections.* Rev Med Virol, 2002. **12**(3): p. 159-66.
114. Wilson, I.A., J.J. Skehel, and D.C. Wiley, *Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution.* Nature, 1981. **289**(5796): p. 366-73.
115. Air, G.M., *Sequence relationships among the hemagglutinin genes of 12 subtypes of influenza A virus.* Proceedings of the National Academy of Sciences, 1981. **78**(12): p. 7639.
116. Eisen, M.B., et al., *Binding of the influenza A virus to cell-surface receptors: structures of five hemagglutinin-sialyloligosaccharide complexes determined by X-ray crystallography.* Virology, 1997. **232**(1): p. 19-31.
117. Rogers, G.N. and B.L. D'Souza, *Receptor binding properties of human and animal H1 influenza virus isolates.* Virology, 1989. **173**(1): p. 317-22.
118. Gambaryan, A.S., J.S. Robertson, and M.N. Matrosovich, *Effects of Egg-Adaptation on the Receptor-Binding Properties of Human Influenza A and B Viruses.* Virology, 1999. **258**(2): p. 232-239.
119. Matrosovich, M.N., et al., *Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site.* Virology, 1997. **233**(1): p. 224-34.
120. Matrosovich, M., et al., *Avian-virus-like receptor specificity of the hemagglutinin impedes influenza virus replication in cultures of human airway epithelium.* Virology, 2007. **361**(2): p. 384-90.
121. Nicholls, J.M., et al., *Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract.* Nature Medicine, 2007. **13**(2): p. 147-149.
122. van Riel, D., et al., *Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals.* Am J Pathol, 2007. **171**(4): p. 1215-23.
123. van Riel, D., et al., *H5N1 Virus Attachment to Lower Respiratory Tract.* Science, 2006. **312**(5772): p. 399.
124. Lin, Y.P., et al., *Evolution of the receptor binding properties of the influenza A(H3N2) hemagglutinin.* Proc Natl Acad Sci U S A, 2012. **109**(52): p. 21474-9.
125. Weis, W., et al., *Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid.* Nature, 1988. **333**(6172): p. 426-31.

126. Gamblin, S.J., et al., *The structure and receptor binding properties of the 1918 influenza hemagglutinin.* Science, 2004. **303**(5665): p. 1838-42.
127. Wilson, I.A., J.J. Skehel, and D.C. Wiley, *Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution.* Nature, 1981. **289**(5796): p. 366-373.
128. Wang, C.-C., et al., *Glycans on influenza hemagglutinin affect receptor binding and immune response.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(43): p. 18137-18142.
129. Skehel, J.J., et al., *A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody.* Proc Natl Acad Sci U S A, 1984. **81**(6): p. 1779-83.
130. Tumpey, T.M., et al., *A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission.* Science, 2007. **315**(5812): p. 655-9.
131. Glaser, L., et al., *A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity.* Journal of virology, 2005. **79**(17): p. 11533-6.
132. Stevens, J., et al., *Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities.* J Mol Biol, 2006. **355**(5): p. 1143-55.
133. Peng, W., et al., *Recent H3N2 Viruses Have Evolved Specificity for Extended, Branched Human-type Receptors, Conferring Potential for Increased Avidity.* Cell Host Microbe, 2017. **21**(1): p. 23-34.
134. Byrd-Leotis, L., et al., *Antigenic Pressure on H3N2 Influenza Virus Drift Strains Imposes Constraints on Binding to Sialylated Receptors but Not Phosphorylated Glycans.* Journal of Virology, 2019. **93**(22): p. e01178-19.
135. Nobusawa, E., et al., *Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses.* Virology, 1991. **182**(2): p. 475-485.
136. Naeve, C.W. and R.G. Webster, *Sequence of the hemagglutinin gene from influenza virus A/Seal/Mass/1/80.* Virology, 1983. **129**(2): p. 298-308.
137. Vines, A., et al., *The role of influenza A virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction.* J Virol, 1998. **72**(9): p. 7626-31.
138. Stevens, J., et al., *Recent avian H5N1 viruses exhibit increased propensity for acquiring human receptor specificity.* J Mol Biol, 2008. **381**(5): p. 1382-94.
139. Tharakaraman, K., et al., *Glycan receptor binding of the influenza A virus H7N9 hemagglutinin.* Cell, 2013. **153**(7): p. 1486-93.
140. Imai, M. and Y. Kawaoka, *The role of receptor binding specificity in interspecies transmission of influenza viruses.* Current opinion in virology, 2012. **2**(2): p. 160-167.
141. Maines, T.R., et al., *Effect of receptor binding domain mutations on receptor binding and transmissibility of avian influenza H5N1 viruses.* Virology, 2011. **413**(1): p. 139-47.
142. Imai, M., et al., *Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets.* Nature, 2012. **486**(7403): p. 420-8.

143. Herfst, S., et al., *Airborne transmission of influenza A/H5N1 virus between ferrets.* Science, 2012. **336**(6088): p. 1534-41.
144. Wang, W., et al., *Glycosylation at 158N of the hemagglutinin protein and receptor binding specificity synergistically affect the antigenicity and immunogenicity of a live attenuated H5N1 A/Vietnam/1203/2004 vaccine virus in ferrets.* J Virol, 2010. **84**(13): p. 6570-7.
145. Chen, J., et al., *Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation.* Cell, 1998. **95**(3): p. 409-17.
146. Russell, R.J., et al., *H1 and H7 influenza haemagglutinin structures extend a structural classification of haemagglutinin subtypes.* Virology, 2004. **325**(2): p. 287-96.
147. Kido, H., et al., *Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. A possible activator of the viral fusion glycoprotein.* J Biol Chem, 1992. **267**(19): p. 13573-9.
148. Garten, W., et al., *Proteolytic activation of the influenza virus hemagglutinin: The structure of the cleavage site and the enzymes involved in cleavage.* Virology, 1981. **115**(2): p. 361-74.
149. Webster, R.G. and R. Rott, *Influenza virus A pathogenicity: the pivotal role of hemagglutinin.* Cell, 1987. **50**(5): p. 665-6.
150. Steinhauer, D.A., *Role of hemagglutinin cleavage for the pathogenicity of influenza virus.* Virology, 1999. **258**(1): p. 1-20.
151. Huang, R.T., R. Rott, and H.D. Klenk, *Influenza viruses cause hemolysis and fusion of cells.* Virology, 1981. **110**(1): p. 243-7.
152. Galloway, S.E., et al., *Influenza HA subtypes demonstrate divergent phenotypes for cleavage activation and pH of fusion: implications for host range and adaptation.* PLoS Pathog, 2013. **9**(2): p. e1003151.
153. Ruigrok, R.W., et al., *Conformational changes in the hemagglutinin of influenza virus which accompany heat-induced fusion of virus with liposomes.* Virology, 1986. **155**(2): p. 484-97.
154. Wharton, S.A., J.J. Skehel, and D.C. Wiley, *Studies of influenza haemagglutinin-mediated membrane fusion.* Virology, 1986. **149**(1): p. 27-35.
155. Chen, J., et al., *A soluble domain of the membrane-anchoring chain of influenza virus hemagglutinin (HA2) folds in Escherichia coli into the low-pH-induced conformation.* Proc Natl Acad Sci U S A, 1995. **92**(26): p. 12205-9.
156. Carr, C.M. and P.S. Kim, *A spring-loaded mechanism for the conformational change of influenza hemagglutinin.* Cell, 1993. **73**(4): p. 823-32.
157. Weber, T., et al., *Evidence for H(+)-induced insertion of influenza hemagglutinin HA2 N-terminal segment into viral membrane.* Journal of Biological Chemistry, 1994. **269**(28): p. 18353-18358.
158. Wharton, S.A., et al., *Electron microscopy of antibody complexes of influenza virus haemagglutinin in the fusion pH conformation.* EMBO J, 1995. **14**(2): p. 240-6.
159. Daniels, R.S., et al., *Fusion mutants of the influenza virus hemagglutinin glycoprotein.* Cell, 1985. **40**(2): p. 431-9.

160. Lin, Y.P., et al., *Adaptation of Egg-Grown and Transfectant Influenza Viruses for Growth in Mammalian Cells: Selection of Hemagglutinin Mutants with Elevated pH of Membrane Fusion.* Virology, 1997. **233**(2): p. 402-410.
161. Thoennes, S., et al., *Analysis of residues near the fusion peptide in the influenza hemagglutinin structure for roles in triggering membrane fusion.* Virology, 2008. **370**(2): p. 403-14.
162. Calder, L.J. and P.B. Rosenthal, *Cryomicroscopy provides structural snapshots of influenza virus membrane fusion.* Nature Structural & Molecular Biology, 2016. **23**(9): p. 853-858.
163. Daniels, P.S., et al., *The receptor-binding and membrane-fusion properties of influenza virus variants selected using anti-haemagglutinin monoclonal antibodies.* EMBO J, 1987. **6**(5): p. 1459-65.
164. Weis, W.I., et al., *The structure of a membrane fusion mutant of the influenza virus haemagglutinin.* EMBO J, 1990. **9**(1): p. 17-24.
165. Koerner, I., et al., *Altered receptor specificity and fusion activity of the haemagglutinin contribute to high virulence of a mouse-adapted influenza A virus.* J Gen Virol, 2012. **93**(Pt 5): p. 970-9.
166. Korte, T., et al., *Role of the Glu Residues of the Influenza Hemagglutinin Fusion Peptide in the pH Dependence of Fusion Activity.* Virology, 2001. **289**(2): p. 353-361.
167. Reed, M.L., et al., *Amino acid residues in the fusion peptide pocket regulate the pH of activation of the H5N1 influenza virus hemagglutinin protein.* J Virol, 2009. **83**(8): p. 3568-80.
168. Langley, W.A., et al., *Single residue deletions along the length of the influenza HA fusion peptide lead to inhibition of membrane fusion function.* Virology, 2009.
169. Byrd-Leotis, L., et al., *Influenza hemagglutinin (HA) stem region mutations that stabilize or destabilize the structure of multiple HA subtypes.* J Virol, 2015. **89**(8): p. 4504-16.
170. Steinhauer, D.A., et al., *Studies of the membrane fusion activities of fusion peptide mutants of influenza virus hemagglutinin.* J Virol, 1995. **69**(11): p. 6643-51.
171. Xu, R. and I.A. Wilson, *Structural characterization of an early fusion intermediate of influenza virus hemagglutinin.* Journal of virology, 2011. **85**(10): p. 5172-5182.
172. Trost, J.F., et al., *A conserved histidine in Group-1 influenza subtype hemagglutinin proteins is essential for membrane fusion activity.* Virology, 2019. **536**: p. 78-90.
173. Steinhauer, D.A., et al., *Studies using double mutants of the conformational transitions in influenza hemagglutinin required for its membrane fusion activity.* Proc Natl Acad Sci U S A, 1996. **93**(23): p. 12873-8.
174. White, J.M. and I.A. Wilson, *Anti-peptide antibodies detect steps in a protein conformational change: low-pH activation of the influenza virus hemagglutinin.* The Journal of cell biology, 1987. **105**(6 Pt 2): p. 2887-2896.
175. Ivanovic, T., et al., *Influenza-virus membrane fusion by cooperative fold-back of stochastically induced hemagglutinin intermediates.* eLife, 2013. **2**: p. e00333-e00333.
176. Yewdell, J.W., W. Gerhard, and T. Bachi, *Monoclonal anti-hemagglutinin antibodies detect irreversible antigenic alterations that coincide with the acid*

activation of influenza virus A/PR/834-mediated hemolysis. Journal of Virology, 1983. **48**(1): p. 239.
177. Daniels, R.S., et al., *Analyses of the antigenicity of influenza haemagglutinin at the pH optimum for virus-mediated membrane fusion.* J Gen Virol, 1983. **64 (Pt 8)**: p. 1657-62.
178. Trost, J.F., et al., *Development of a high-throughput assay to detect antibody inhibition of low pH induced conformational changes of influenza virus hemagglutinin.* PloS one, 2018. **13**(6): p. e0199683-e0199683.
179. Hovanec, D.L. and G.M. Air, *Antigenic structure of the hemagglutinin of influenza virus B/Hong Kong/8/73 as determined from gene sequence analysis of variants selected with monoclonal antibodies.* Virology, 1984. **139**(2): p. 384-92.
180. Tsuchiya, E., et al., *Antigenic structure of the haemagglutinin of human influenza A/H2N2 virus.* Journal of General Virology, 2001. **82**(10): p. 2475-2484.
181. Kida, H., et al., *Interference with a conformational change in the haemagglutinin molecule of influenza virus by antibodies as a possible neutralization mechanism.* Vaccine, 1985. **3**(3): p. 219-222.
182. Knossow, M., et al., *Mechanism of neutralization of influenza virus infectivity by antibodies.* Virology, 2002. **302**(2): p. 294-8.
183. Ekiert, D.C., et al., *Antibody recognition of a highly conserved influenza virus epitope.* Science (New York, N.Y.), 2009. **324**(5924): p. 246-251.
184. Dreyfus, C., D.C. Ekiert, and I.A. Wilson, *Structure of a classical broadly neutralizing stem antibody in complex with a pandemic H2 influenza virus hemagglutinin.* Journal of virology, 2013. **87**(12): p. 7149-7154.
185. Mair, C.M., et al., *Receptor binding and pH stability - how influenza A virus hemagglutinin affects host-specific virus infection.* Biochim Biophys Acta, 2014. **1838**(4): p. 1153-68.
186. DuBois, R.M., et al., *Acid stability of the hemagglutinin protein regulates H5N1 influenza virus pathogenicity.* PLoS Pathog, 2011. **7**(12): p. e1002398.
187. Reed, M.L., et al., *The pH of activation of the hemagglutinin protein regulates H5N1 influenza virus pathogenicity and transmissibility in ducks.* J Virol, 2010. **84**(3): p. 1527-35.
188. Russier, M., et al., *H1N1 influenza viruses varying widely in hemagglutinin stability transmit efficiently from swine to swine and to ferrets.* PLoS pathogens, 2017. **13**(3): p. e1006276-e1006276.
189. Russier, M., et al., *Molecular requirements for a pandemic influenza virus: An acid-stable hemagglutinin protein.* Proceedings of the National Academy of Sciences, 2016. **113**(6): p. 1636.
190. England, R.J.A., et al., *Nasal pH measurement: a reliable and repeatable parameter.* Clinical Otolaryngology & Allied Sciences, 1999. **24**(1): p. 67-68.
191. Brown, J.D., et al., *Avian influenza virus in water: infectivity is dependent on pH, salinity and temperature.* Vet Microbiol, 2009. **136**(1-2): p. 20-6.
192. Garten, R.J., et al., *Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans.* Science, 2009. **325**(5937): p. 197-201.

193. Zhang, Y., et al., *Key Molecular Factors in Hemagglutinin and PB2 Contribute to Efficient Transmission of the 2009 H1N1 Pandemic Influenza Virus.* Journal of Virology, 2012. **86**(18): p. 9666.
194. Gubareva, L.V., R.G. Webster, and F.G. Hayden, *Comparison of the activities of zanamivir, oseltamivir, and RWJ-270201 against clinical isolates of influenza virus and neuraminidase inhibitor-resistant variants.* Antimicrob Agents Chemother, 2001. **45**(12): p. 3403-8.
195. Hayden, F., et al., *Neuraminidase inhibitor susceptibility network position statement: antiviral resistance in influenza A/H5N1 viruses.* Antivir Ther, 2005. **10**(8): p. 873-7.
196. Hayden, F.G., et al., *Baloxavir Marboxil for Uncomplicated Influenza in Adults and Adolescents.* N Engl J Med, 2018. **379**(10): p. 913-923.
197. Bodian, D.L., et al., *Inhibition of the fusion-inducing conformational change of influenza hemagglutinin by benzoquinones and hydroquinones.* Biochemistry, 1993. **32**(12): p. 2967-78.
198. Hoffman LR, K.I., White JM, *tructure-based identification of an inducer of the low-pH conformational change in the influenza virus hemagglutinin: irreversible inhibition of infectivity.* J Virol., 1997. **71**(11): p. 8808-8820.
199. Russell, R.J., et al., *Structure of influenza hemagglutinin in complex with an inhibitor of membrane fusion.* Proc Natl Acad Sci U S A, 2008. **105**(46): p. 17736-41.
200. Fleishman, S.J., et al., *Computational design of proteins targeting the conserved stem region of influenza hemagglutinin.* Science (New York, N.Y.), 2011. **332**(6031): p. 816-821.
201. Throsby, M., et al., *Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells.* PloS one, 2008. **3**(12): p. e3942-e3942.
202. Corti, D., et al., *A Neutralizing Antibody Selected from Plasma Cells That Binds to Group 1 and Group 2 Influenza A Hemagglutinins.* Science, 2011. **333**(6044): p. 850.
203. Sui, J., et al., *Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses.* Nature structural & molecular biology, 2009. **16**(3): p. 265-273.
204. van Dongen, M.J.P., et al., *A small-molecule fusion inhibitor of influenza virus is orally active in mice.* Science, 2019. **363**(6431): p. eaar6221.
205. Lazarowitz, S.G. and P.W. Choppin, *Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide.* Virology, 1975. **68**(2): p. 440-54.
206. Bullough, P.A., et al., *Structure of influenza haemagglutinin at the pH of membrane fusion.* Nature, 1994. **371**(6492): p. 37-43.
207. Bizebard, T., et al., *Structure of influenza virus haemagglutinin complexed with a neutralizing antibody.* Nature, 1995. **376**(6535): p. 92-4.
208. Stevens, J., et al., *Structure of the Uncleaved Human H1 Hemagglutinin from the Extinct 1918 Influenza Virus.* Science, 2004. **303**(5665): p. 1866.

209. Lu, X., et al., *Insights into Avian Influenza Virus Pathogenicity: the Hemagglutinin Precursor HA0 of Subtype H16 Has an Alpha-Helix Structure in Its Cleavage Site with Inefficient HA1/HA2 Cleavage.* Journal of Virology, 2012. **86**(23): p. 12861.
210. Ha, Y., et al., *H5 avian and H9 swine influenza virus haemagglutinin structures: possible origin of influenza subtypes.* EMBO J, 2002. **21**(5): p. 865-75.
211. Liu, J., et al., *Structures of receptor complexes formed by hemagglutinins from the Asian Influenza pandemic of 1957.* Proc Natl Acad Sci U S A, 2009. **106**(40): p. 17175-80.
212. Cross, K.J., et al., *Studies on influenza haemagglutinin fusion peptide mutants generated by reverse genetics.* EMBO J, 2001. **20**(16): p. 4432-42.
213. Cross, K.J., et al., *Composition and functions of the influenza fusion peptide.* Protein Pept Lett, 2009. **16**(7): p. 766-78.
214. Gething, M.J., et al., *Studies on the mechanism of membrane fusion: site-specific mutagenesis of the hemagglutinin of influenza virus.* J Cell Biol, 1986. **102**(1): p. 11-23.
215. Lin, X., et al., *Lowered pH Leads to Fusion Peptide Release and a Highly Dynamic Intermediate of Influenza Hemagglutinin.* The journal of physical chemistry. B, 2016. **120**(36): p. 9654-9660.
216. Yewdell, J.W., et al., *Mutations in or near the fusion peptide of the influenza virus hemagglutinin affect an antigenic site in the globular region.* Journal of virology, 1993. **67**(2): p. 933-942.
217. Ekiert, D.C., et al., *A highly conserved neutralizing epitope on group 2 influenza A viruses.* Science (New York, N.Y.), 2011. **333**(6044): p. 843-850.
218. Wu, Y., et al., *A potent broad-spectrum protective human monoclonal antibody crosslinking two haemagglutinin monomers of influenza A virus.* Nature communications, 2015. **6**: p. 7708-7708.
219. Kallewaard, N.L., et al., *Structure and Function Analysis of an Antibody Recognizing All Influenza A Subtypes.* Cell, 2016. **166**(3): p. 596-608.
220. Qiao, H., et al., *A specific point mutant at position 1 of the influenza hemagglutinin fusion peptide displays a hemifusion phenotype.* Molecular biology of the cell, 1999. **10**(8): p. 2759-2769.
221. Dawkins, A.T., Jr., et al., *Studies on Induced Influenza in Man: II. Double-Blind Study Designed to Assess the Prophylactic Efficacy of an Analogue of Amantadine Hydrochloride.* JAMA, 1968. **203**(13): p. 1095-1099.
222. Dolin, R., et al., *A Controlled Trial of Amantadine and Rimantadine in the Prophylaxis of Influenza a Infection.* New England Journal of Medicine, 1982. **307**(10): p. 580-584.
223. Hay, A.J., et al., *Molecular basis of resistance of influenza A viruses to amantadine.* J Antimicrob Chemother, 1986. **18 Suppl B**: p. 19-29.
224. Deyde, V.M., et al., *Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide.* J Infect Dis, 2007. **196**(2): p. 249-57.
225. Dutkowski, R., *Oseltamivir in seasonal influenza: cumulative experience in low- and high-risk patients.* Journal of Antimicrobial Chemotherapy, 2010. **65**(suppl_2): p. ii11-ii24.

226. von Itzstein, M., et al., *Rational design of potent sialidase-based inhibitors of influenza virus replication.* Nature, 1993. **363**(6428): p. 418-423.
227. Woods, J.M., et al., *4-Guanidino-2,4-dideoxy-2,3-dehydro-N-acetylneuraminic acid is a highly effective inhibitor both of the sialidase (neuraminidase) and of growth of a wide range of influenza A and B viruses in vitro.* Antimicrobial Agents and Chemotherapy, 1993. **37**(7): p. 1473.
228. Gubareva, L.V., et al., *Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus.* J Infect Dis, 1998. **178**(5): p. 1257-62.
229. Grohskopf, L.A., et al., *Prevention and Control of Seasonal Influenza with Vaccines: Recommendations of the Advisory Committee on Immunization Practices - United States, 2019-20 Influenza Season.* MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports, 2019. **68**(3): p. 1-21.
230. Lamb, Y.N., *Cell-Based Quadrivalent Inactivated Influenza Virus Vaccine (Flucelvax(®) Tetra/Flucelvax Quadrivalent(®)): A Review in the Prevention of Influenza.* Drugs, 2019. **79**(12): p. 1337-1348.
231. Tisa, V., et al., *Quadrivalent influenza vaccine: a new opportunity to reduce the influenza burden.* Journal of preventive medicine and hygiene, 2016. **57**(1): p. E28-E33.
232. Barr, I.G., et al., *Epidemiological, antigenic and genetic characteristics of seasonal influenza A(H1N1), A(H3N2) and B influenza viruses: Basis for the WHO recommendation on the composition of influenza vaccines for use in the 2009–2010 Northern Hemisphere season.* Vaccine, 2010. **28**(5): p. 1156-1167.
233. Corti, D., et al., *Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine.* The Journal of clinical investigation, 2010. **120**(5): p. 1663-1673.
234. Steel, J., et al., *Influenza virus vaccine based on the conserved hemagglutinin stalk domain.* mBio, 2010. **1**(1): p. e00018-10.
235. Bommakanti, G., et al., *Design of an HA2-based Escherichia coli expressed influenza immunogen that protects mice from pathogenic challenge.* Proceedings of the National Academy of Sciences, 2010. **107**(31): p. 13701.
236. Wrammert, J., et al., *Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection.* The Journal of Experimental Medicine, 2011. **208**(1): p. 181-193.
237. Wang, T.T., et al., *Broadly Protective Monoclonal Antibodies against H3 Influenza Viruses following Sequential Immunization with Different Hemagglutinins.* PLOS Pathogens, 2010. **6**(2): p. e1000796.
238. Okuno, Y., et al., *A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains.* Journal of virology, 1993. **67**(5): p. 2552-2558.
239. Tharakaraman, K., et al., *Broadly neutralizing influenza hemagglutinin stem-specific antibody CR8020 targets residues that are prone to escape due to host selection pressure.* Cell host & microbe, 2014. **15**(5): p. 644-651.

240. Friesen, R.H.E., et al., *A common solution to group 2 influenza virus neutralization.* Proceedings of the National Academy of Sciences, 2014. **111**(1): p. 445.
241. Kinder, J.T., et al., *Human metapneumovirus fusion protein triggering: Increasing complexities by analysis of new HMPV fusion proteins.* Virology, 2019. **531**: p. 248-254.
242. Wu, W., et al., *Mapping the pH Sensors Critical for Host Cell Entry by a Complex Nonenveloped Virus.* Journal of virology, 2019. **93**(4): p. e01897-18.
243. Chaudhury, S., D.R. Ripoll, and A. Wallqvist, *Structure-based pKa prediction provides a thermodynamic basis for the role of histidines in pH-induced conformational transitions in dengue virus.* Biochemistry and biophysics reports, 2015. **4**: p. 375-385.
244. Russell, C.J., M. Hu, and F.A. Okda, *Influenza Hemagglutinin Protein Stability, Activation, and Pandemic Risk.* Trends in Microbiology, 2018. **26**(10): p. 841-853.
245. Pezzulo, A.A., et al., *Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung.* Nature, 2012. **487**(7405): p. 109-113.