**SUPPLEMENTAL MATERIALS AND METHODS**

**Viruses.** A nasal swab from a Golden Retriever displaying signs of respiratory illness was collected in virus transport media. The sample was inoculated into embryonated chicken eggs and the harvested allantoic fluid was blind passaged in Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) and virus was isolated. Virus stocks of A/canine/IL/12191/15 (H3N2) were then generated by passage in the allantoic cavity of 10-day-old embryonated hens’ eggs at 35°C for 48h. Full length ORF sequence analysis of each of the eight vRNAs extracted from the egg passaged stock confirmed that the stock was 100% identical to the genomic sequence of the egg/cell propagated seed virus (GenBank accession number KT002533-KT002540). A/Switzerland/9715293/13 (H3N2) virus was propagated in either MDCK cells (antigen used for HI testing) or the allantoic cavity of 10-day-old embryonated hens’ eggs at 35°C for 48h (for replication kinetic studies due to infectivity titer required). Allantoic fluid was pooled from multiple eggs, clarified by centrifugation, and frozen in aliquots at -80°C. The seasonal H1N1 virus, A/Brisbane/59/07, was propagated in MDCK cells at 37°C for 48 hrs. Per institutional biosafety requirements, exclusivity tests were performed prior to animal experiments to rule out the presence of other subtypes of IAVs in each virus stock. To determine the 50% egg infectious dose (EID50) for each virus stock, eggs were inoculated with serially diluted virus and the EID50 was calculated using the Reed and Muench method [1].

**Recombinant HAs and NAs cloning and expression.** The cDNAs for the ectodomains of the A/canine/IL/11613/2015 (H3N2) HA (residues 1-503 in mature protein numbering) and NA (residues 80-470) were synthesized (GenScript USA Inc.) as codon optimized genes for insect cell expression, and sub-cloned into the baculovirus transfer vector, pAcGP67B (BD Biosciences). To aid purification, the HA had an additional C-terminal thrombin site followed by a foldon trimerization sequence from the bacteriophage T4 fibritin and a His-Tag incorporated into the final construct [2]. The recombinant NA protein contained an N-terminal His-tag, tetramerization domain from the human vasodilator-stimulated phosphoprotein [3] and a thrombin cleavage site [4]. Secreted proteins were recovered from the culture supernatant and purified by metal affinity chromatography and size exclusion chromatography (SEC). For structural analyses, proteins were further subjected to trypsin cleavage and SEC. Trypsin-treated proteins were buffer exchanged into 10 mM Tris-HCl, 50 mM NaCl, pH 8.0 and concentrated for crystallization trials.

**Crystallization and structure determination.** Initial crystallization trials were set up for the HA and NA of A/canine/IL/11613/2015 virus using Oryx4, a crystallization robot for sitting drop (Douglas Instruments Ltd, Berkshire, UK). Conditions in which crystals were observed were optimized at 20°C using a modified method for microbatch under oil [5]. Both HA and NA were crystallized in 0.2M calcium acetate, 0.1M Tris pH 7.0, and 20% (w/v) PEG 3000. HA crystals were flash-cooled at 100 K using 20% glycerol as cryoprotectant while NA crystals were flashed cooled at 100 K without any cryoprotectant. The datasets were collected and processed with the DENZO-SACLEPACK suite [6].

**Structure determination and refinement.** The HA and NA structures of A/canine/IL/11613/2015 virus were determined by molecular replacement with Phaser [7]. For the HA, the A(H3N8) A/harbor seal/Massachusetts/1/2011structure (PDB: 4WA1) was used as a search model [8]. For the NA, the 1957 pandemic (H2N2) structure (PDB: 3TIA) was used as a search model [9]. The sequence for the model was then mutated to the correct sequence, rebuilt by Coot [10], and refined with REFMAC [11] using both TLS refinement [12] and Phenix refine [13]. All final models were assessed using MolProbity [14] and statistics for data processing and refinement are presented in Supplemental Table 1. All structural figures were generated with MacPyMol [15].

**Mouse experiments.** Animal experiments were performed under the guidance of the CDC Institutional Animal Care and Use Committee and were conducted in a BSL-3E laboratory accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Six to eight week old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were anesthetized intraperitoneally with 0.2 ml of 2,2,2-tribromoethanol in tert-amyl alcohol (Avertin; Acros Organics) and inoculated intranasally (i.n.) with 50µl of A/canine/IL/12191/2015 virus diluted in phosphate-buffered saline (PBS). Fifty percent mouse infectious dose (MID50) was determined by inoculating groups of 3 mice with 107.2 EID50 and serial 10-fold dilutions of virus ranging from 107.0 to 101.0 EID50 [16]. Mice from each group were euthanized on day 3 post-inoculation (p.i.) and the lungs were collected to determine viral titers using the Reed and Muench method [1]. Additional groups of five mice inoculated with 107.2 and 106.0 EID50 were monitored for 14 days for clinical signs of infection and weight loss.

**Ferret experiments**. Nine-month old, male Fitch ferrets (Triple F Farms, Sayre, PA) were housed in Duo-Flo Bioclean mobile units (Lab Products Incorporated, Seaford, DE) during the study. Prior to the study, serum from each of the ferrets was analyzed by hemagglutination inhibition assay to confirm that each animal was serologically negative for currently circulating IAVs and the CIVs included in this study. Three ferrets each were inoculated i.n. with 107.1 EID50 of A/canine/IL/12191/2015 virus diluted in PBS. The following day, a serologically naive ferret was placed in the same cage as each inoculated ferret for the assessment of virus transmission between ferrets in direct contact [17]. The co-housed pairs of ferrets were observed daily for clinical signs of infection, and nasal washes and rectal swabs were collected every two days. Three additional ferrets were inoculated with 107.1 EID50 of virus and then euthanized on day 3 p.i. for the assessment of virus replication and systemic spread, as previously described [16].

**SUPPLEMENTAL Figures**

**S Figure 1. PB2**



**S Figure 2. PB1**



**S Figure 3. PA**



**S Figure 4. NP**



**S Figure 5. NA**





**S Figure 6. M**



**S Figure 7. NS**

**Supplemental Figures 1-7.** Phylogenetic trees of the NA and internal genes of Eurasian lineage avian and H3N2 CIVs generated using a general time reversible model and maximum likelihood method with 1000 bootstrap replicates. Bootstraps of 60 and greater are shown at branch nodes. The scale bar represents nucleotide substitutions per site.

**SUPPLEMENTAL TABLES**

**Supplemental Table 1. Data collection and refinement statistics for CIV HA and NA crystal structures.**

|  |  |  |
| --- | --- | --- |
|  | **HA** | **NA** |
| **Data collection** |  |  |
| Space group | R3 | P21212 |
| Cell dimensions |  |  |
| *a*, *b*, *c* (Å) | 241.15,241.15,147.96 | 110.45,110.56,126.62 |
| Cell angle (°) | 90, 90, 120 | 90, 90, 90 |
| Resolution (Å) | 50-3.0 (3.12-3.01)a | 50-1.8 (1.86-1.80) |
| *R*sym | 0.113 (0.691) | 0.113 (0.623) |
| I/σ | 17.3 (1.7) | 25.0 (2.3) |
| Completeness (%) | 99.0 (98.8) | 99.9 (99.0) |
| Redundancy | 3.7 (3.6) | 7.2 (6.2) |
|  |  |  |
| **Refinement** |  |  |
| Resolution (Å) | 50-3.0 (3.12-3.01) | 50-1.8 (1.86-1.80) |
| No. reflections | 62940 (6197) | 143267 (13889) |
| *R*work / *R*free | 21.3/24.2 | 25.3/27.8 |
| No. atoms |  |  |
| Protein | 15352 | 12404 |
| Ligand/ion | 0 | 460 |
| *B*-factors |  |  |
| Protein | 104.6 | 27.0 |
| Ligand/ion | N/A | 49.3 |
| R.m.s. deviations |  |  |
| Bond lengths (Å) | 0.012 | 0.016 |
| Bond angles (°) | 1.48 | 1.78 |

a Numbers in parentheses refer to the highest resolution shell.

N/A – not applicable

**Supplemental Table 2. Glycan microarray for CIV HA.**

|  |  |  |
| --- | --- | --- |
| **#** | **Structure** | **A/canine/IL/11613/15** |
| 1 | Neu5Acα | nb |
| 2 | Neu5Acα | nb |
| 3 | Neu5Acβ | nb |
| 4 | Neu5Acα2-3(6-O-Su)Galβ1-4GlcNAcβ | +++ |
| 5 | Neu5Acα2-3Galβ1-3[6OSO3]GalNAcα | +++ |
| 6 | Neu5Acα2-3Galβ1-4[6OSO3]GlcNAcβ | +++ |
| 7 | Neu5Acα2-3Galβ1-4(Fucα1-3)[6OSO3]GlcNAcβ-propyl-NH2 | +++ |
| 8 | Neu5Acα2-3Galβ1-3[6OSO3]GlcNAc-propyl-NH2 | +++ |
| 9 | Neu5Acα2-3Galβ1-3(Neu5Acα2-3Galβ1-4)GlcNAcβ | +++ |
| 10 | Neu5Acα2-3Galβ1-3(Neu5Acα2-3Galβ1-4GlcNAcβ1-6)GalNAcα | +++ |
| 11 | Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ | +++ |
| 12 | Neu5Acα(2-3)-Galβ(1-4)-GlcNAcβ(1-3)-Galβ(1-4)-GlcNAcβ(1-2)-Manα(1-3)-[Neu5Acα(2-3)-Galβ(1-4)-GlcNAcβ(1-3)-Galβ(1-4)-GlcNAcβ(1-2)-Manα(1-6)]-Manβ(1-4)-GlcNAcβ(1-4)-GlcNAcβ | +++ |
| 13 | Neu5Acα2-3Galβ | +++ |
| 14 | Neu5Acα2-3Galβ1-3GalNAcα | +++ |
| 15 | Neu5Acα2-3Galβ1-3GlcNAcβ | +++ |
| 16 | Neu5Acα2-3Galβ1-3GlcNAcβ | +++ |
| 17 | Neu5Acα2-3Galβ1-4Glcβ | +++ |
| 18 | Neu5Acα2-3Galβ1-4Glcβ | +++ |
| 19 | Neu5Acα2-3Galβ1-4GlcNAcβ | +++ |
| 20 | Neu5Acα2-3Galβ1-4GlcNAcβ | +++ |
| 21 | Neu5Acα2-3GalNAcβ1-4GlcNAcβ | nb |
| 22 | Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ | +++ |
| 23 | Neu5Aca2-3Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ | +++ |
| 24 | Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ | +++ |
| 25 | Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-3GlcNAcβ | +++ |
| 26 | Neu5Acα2-3Galβ1-3GalNAcα | +++ |
| 27 | Galβ1-3(Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-6)GalNAcα | + |
| 28 | Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβ | +++ |
| 29 | Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ | +++ |
| 30 | Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ | +++ |
| 31 | Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ | +++ |
| 32 | Neu5Acα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4[Fucα1-3]GlcNAcβ | +++ |
| 33 | Neu5Acα2-3Galβ1-3[Fucα1-3]GlcNAcβ1-3Galβ1-4[Fucα1-3]GlcNAcβ | +++ |
| 34 | Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ | +++ |
| 35 | Neu5Acα2-3(GalNAcβ1-4)Galβ1-4GlcNAcβ | nb |
| 36 | Neu5Acα2-3(GalNAcβ1-4)Galβ1-4GlcNAcβ | nb |
| 37 | Neu5Acα2-3(GalNAcβ1-4)Galβ1-4Glcβ | nb |
| 38 | Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ | nb |
| 39 | Fucα1-2Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ | nb |
| 40 | Fucα1-2Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ | nb |
| 41 | Neu5Acα2-6Galβ1-4[6OSO3]GlcNAcβ | nb |
| 42 | Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ | nb |
| 43 | Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ | nb |
| 44 | Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3[Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAcβ | nb |
| 45 | Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3[Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6]-Manβ1-4GlcNAcβ1-4GlcNAcβ | + |
| 46 | Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3[Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6]GalNAca | nb |
| 47 | Neu5Acα2-6Galβ1-4GlcNAcβ1-3[Neu5Acα2-6Galβ1-4GlcNAcβ1-6]GalNAca | nb |
| 48 | Neu5Acα2-6GalNAcα | nb |
| 49 | Neu5Acα2-6Galβ | nb |
| 50 | Neu5Acα2-6Galβ1-4Glcβ | nb |
| 51 | Neu5Acα2-6Galβ1-4Glcβ | nb |
| 52 | Neu5Acα2-6Galβ1-4GlcNAcβ | nb |
| 53 | Neu5Acα2-6Galβ1-4GlcNAcβ | nb |
| 54 | Neu5Acα2-6GalNAcβ1-4GlcNAcβ | nb |
| 55 | Neu5Acα2-6Galβ1-4GlcNAcβ1-3GalNAcα | nb |
| 56 | Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ | nb |
| 57 | Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3GalNAcα | nb |
| 58 | Neu5Aca2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ | nb |
| 59 | Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ | nb |
| 60 | Galβ1-3(Neu5Acα2-6)GlcNAcβ1-4Galβ1-4Glcβ-Sp10 | nb |
| 61 | Neu5Acα2-6[Galβ1-3]GalNAca | nb |
| 62 | Neu5Acα2-6Galβ1-4GlcNAcβ1-6[Galβ1-3]GalNAca | nb |
| 63 | Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6[Galβ1-3]GalNAca | nb |
| 64 | Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ | nb |
| 65 | Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ | +++ |
| 66 | Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAcα | +++ |
| 67 | Neu5Acα2-3(Neu5Acα2-6)GalNAcα | nb |
| 68 | Neu5Gcα | nb |
| 69 | Neu5Gcα2-3Galβ1-3(Fucα1-4)GlcNAcβ | nb |
| 70 | Neu5Gcα2-3Galβ1-3GlcNAcβ | nb |
| 71 | Neu5Gcα2-3Galβ1-4(Fucα1-3)GlcNAcβ | +++ |
| 72 | Neu5Gcα2-3Galβ1-4GlcNAcβ | nb |
| 73 | Neu5Gcα2-6GalNAcα | nb |
| 74 | Neu5Gcα2-6Galβ1-4GlcNAcβ | nb |
| 75 | Neu5Acα2-8Neu5Acα | nb |
| 76 | Neu5Acα2-8Neu5Acα2-8Neu5Acα | nb |
| 77 | Neu5Acα2-8Neu5Acα2-3(GalNAcβ1-4)Galβ1-4Glcβ | nb |
| 78 | Neu5Acα2-8Neu5Acα2-3Galβ1-4Glcβ | nb |
| 79 | Neu5Acα2-8Neu5Acα2-8Neu5Acα2-3(GalNAcβ1-4)Galβ1-4Glcβ | nb |
| 80 | Neu5Acα2-8Neu5Acα2-8Neu5Acα2-3Galβ1-4Glcβ | nb |
| 81 | Neu5Acα2-8Neu5Acβ-Sp17 | nb |
| 82 | Neu5Acα2-8Neu5Acα2-8Neu5Acβ | nb |
| 83 | Neu5Acβ2-6GalNAcα | nb |
| 84 | Neu5Acβ2-6Galβ1-4GlcNAcβ | nb |
| 85 | Neu5Gcβ2-6Galβ1-4GlcNAc | nb |
| 86 | Galβ1-3(Neu5Acβ2-6)GalNAcα | nb |
| 87 | [9NAc]Neu5Acα | nb |
| 88 | [9NAc]Neu5Acα2-6Galβ1-4GlcNAcβ | nb |
| 89 | Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ | nb |
| 90 | Galβ1-3GlcNAcβ1-3Galβ1-3GlcNAcβ | nb |
| 91 | Galβ1-4GlcNAcβ1-2Manα1-3[Galβ1-4GlcNAcβ1-2Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAcβ | nb |
| 92 | GalNAcα1-3(Fucα1-2)Galβ1-3GlcNAcβ | nb |
| 93 | GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ | nb |
| 94 | Galα1-3(Fucα1-2)Galβ1-3GlcNAcβ | nb |
| 95 | Galα1-3(Fucα1-2)Galβ1-4(Fucα1-3)GlcNAcβ | nb |
| 96 | Galβ1-3GalNAcα | nb |

The color coding in the left hand column is the same color scheme used in Figure 2C. Significant binding of samples to glycans were qualitatively estimated based on relative strength of the signal for the data shown in the figure; Fluorescence Intensity >3000 (+++), 2000-2999 (++), 1000-1999 (+), <1000 (nb). Different categories of glycans on the array are color-coded in column 1 as follows: No color, sialic acid; blue, α2-3 sialosides; red, α2-6 sialosides, violet, mixed α2-3/ α2-6 biantennaries; green, N-glycolylneuraminic acid-containing glycans; brown, α2-8 linked sialosides; pink, β2-6 linked and 9-O-acetylated sialic acids; grey, asialo glycans. Key: Neu5Ac = Sialic acid; Neu5Gc = N-glycolylneuraminic acid; OSO3 = sulfate; Gal = galactose; Fuc = fucose; Glc = D-glucose; GlcNAc = N-Acetyl-D-glucosamine; GalNAc = N-acetyl-D-galactosamine; Man = D-mannose; 9NAc = 9-O-acetyl.

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