

1 H7 influenza A viruses bind sialyl-LewisX, a  
2 potential intermediate receptor between  
3 species

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5 Running title: H7 IAVs bind sLe<sup>X</sup>, a potential inter-species receptor

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## 28 **Abstract**

29 Influenza A viruses (IAVs) can overcome species barriers by adaptation of the receptor  
30 binding site of the hemagglutinin (HA). To initiate infection, HAs bind to glycan  
31 receptors with terminal sialic acids, which are either *N*-acetylneuraminic acid (NeuAc)  
32 or *N*-glycolylneuraminic acid (NeuGc), the latter is mainly found in horses and pigs but  
33 not in birds and humans. We investigated the influence of previously identified equine  
34 NeuGc-adapting mutations (S128T, I130V, A135E, T189A, and K193R) in avian H7  
35 IAVs *in vitro* and *in vivo*. We observed that these mutations negatively affected viral  
36 replication in chicken cells, but not in duck cells, and positively affected replication in  
37 horse cells. *In vivo*, the mutations reduced virus virulence and mortality in chickens.  
38 Ducks excreted high viral loads for a longer time than chickens, although they  
39 appeared clinically healthy. To elucidate why chickens and ducks were infected by  
40 these viruses despite the absence of NeuGc, we re-evaluated the receptor binding of  
41 H7 HAs using glycan microarray and flow cytometry studies. This revealed that  
42 mutated avian H7 HAs also bound to  $\alpha$ 2,3-linked NeuAc and sialyl-LewisX, which have  
43 an additional fucose moiety in their terminal epitope, explaining why infection of ducks  
44 and chickens was possible. Interestingly, the  $\alpha$ 2,3-linked NeuAc and sialyl-LewisX  
45 epitopes were only bound when presented on tri-antennary *N*-glycans, emphasizing  
46 the importance of investigating the fine receptor specificities of IAVs. In conclusion,  
47 the binding of NeuGc-adapted H7 IAV to sialyl-LewisX enables viral replication and  
48 shedding by chickens and ducks, potentially facilitating interspecies transmission of  
49 equine-adapted H7 IAVs. (249 words)

## 50 **Importance**

51 Influenza A viruses cause millions of deaths and illness in birds and mammals each  
52 year. The viral surface protein hemagglutinin initiates infection by binding to host cell  
53 terminal sialic acids. Hemagglutinin adaptations affect the binding affinity to these  
54 sialic acids and therefore the potential host species targeted. While avian and human  
55 IAVs tend to bind *N*-acetylneuraminic acid (a form of sialic acid), equine H7 viruses  
56 prefer binding to *N*-glycolylneuraminic acid (NeuGc). To better understand the function  
57 of NeuGc-specific adaptations in hemagglutinin and to elucidate interspecies  
58 transmission potential NeuGc-adapted viruses, we evaluated the effects of NeuGc-  
59 specific mutations in avian H7 viruses in chickens and ducks, important economic  
60 hosts and reservoir birds, respectively. We also examined the impact on viral  
61 replication and found a binding affinity to sialyl-LewisX, another terminal epitope.  
62 These findings are important as they contribute to the understanding of the role of  
63 sialyl-LewisX in avian influenza infection. (148 words)

64

## 65 **Keywords**

66 Influenza A virus, hemagglutinin, interspecies transmission, sialyl-LewisX, NeuGc

## 67 **Introduction**

68 Influenza A viruses (IAVs) are a member of the virus family *Orthomyxoviridae* and their  
69 proteins are encoded on eight single-stranded negative-sense RNA segments with a  
70 total length of 12-14kb (1). The enveloped virion of IAVs is coated with the surface  
71 proteins hemagglutinin (HA) and neuraminidase, which allow the classification into  
72 different subtypes (HxNx). IAVs infect a variety of avian and mammalian species,  
73 including humans, pigs, and horses (2). The natural reservoirs for IAVs are wild  
74 waterfowl, but transmission from ducks to other susceptible avian and mammalian  
75 species is frequent (3, 4). Avian IAV infection in wild birds is often asymptomatic, due  
76 to the coevolution of IAV and wild birds (5). However, in poultry low pathogenicity avian  
77 influenza viruses (LPAIV) can evolve into high pathogenicity avian influenza virus  
78 (HPAIV) causing mortality rates up to 100% in infected flocks. One of the key  
79 determinants for the virulence and pathogenicity of HPAIV is the acquisition of a  
80 multibasic cleavage site in the HA, which is most common in H5 and H7 IAVs (6).

81

82 High pathogenicity H7 IAVs are occasionally transmitted to humans and other  
83 mammalian species (7-9). Equine H7N7 influenza A viruses contain a multibasic  
84 cleavage site and are suspected to have originated from an avian H7 ancestor virus  
85 from an HPAIV outbreak in poultry (10). Furthermore, reassortant viruses with the  
86 equine H7N7 HA and other genes from a chicken H5N2 IAV were shown to be lethal  
87 in chickens (11). Nowadays, equine H7N7 viruses are thought to be extinct, leaving  
88 equine H3N8 as the only active circulating equine influenza virus (12, 13). The  
89 presence of H7 IAVs in different species emphasizes the relevance of further  
90 investigating these viruses and their interspecies transmission.

91

92 Overcoming host species barriers and establishing species-specific influenza strains  
93 involves the accumulation of point mutations during adaptation (14-17). The main host  
94 species barrier of IAVs is the receptor binding specificity of HA to terminal sialic acid  
95 (Sia) epitopes on the host cell surface, which is important for virus uptake into the cell  
96 (18). Receptor binding of HAs is strain-specific and has co-evolved with receptors  
97 found in the respiratory and/or intestinal tract of susceptible host species. Therefore,  
98 avian influenza viruses (AIV) bind preferentially to  $\alpha$ 2,3-linked Sia, whereas human-  
99 adapted strains prefer  $\alpha$ 2,6-linked Sia receptors (7, 19-21).

100

101 Besides the glycosidic linkage, the host cell receptor's type of terminating Sia and the  
102 underlying glycan structures are important factors in IAV receptor binding properties  
103 and host range (14, 22-25). Unlike the majority of IAV, which predominantly bind to  
104 glycans with a terminal *N*-acetylneuraminic acid (NeuAc), equine H7N7 IAV  
105 predominantly bind to the *N*-glycolylneuraminic acid (NeuGc) (26, 27). Levels of  
106 NeuGc are variably present in the respiratory tract of most mammalian species,  
107 especially horses and pigs. However, no NeuGc is expressed in, among others, birds,  
108 humans, and ferrets (28-32). Previously, we identified five mutations S128T, I130V,  
109 A135E, T189A, and K193R in the receptor binding site (RBS), based on an equine  
110 H7N7 virus, that switched avian H7 IAVs from binding NeuAc to NeuGc (33).

111

112 In this study, we examined the impact of the equine NeuGc-adapting mutations  
113 (S128T, I130V, A135E, T189A, and K193R) on avian H7 IAVs both *in vitro* and *in vivo*,  
114 with particular emphasis on economically important poultry (chickens) and natural  
115 reservoir bird (ducks). The mutated viruses showed reduced replication in chicken  
116 cells, however, the replication in duck cells remained unaffected. On the other hand,  
117 viral replication in horse cells was increased. *In vivo*, the NeuGc-adapted viruses  
118 showed reduced mortality and virulence in infected chickens compared to the WT  
119 HPAIV, but the viral distribution between chicken organs was mostly unaffected by the  
120 mutations. However, virus shedding was higher in cloacal swab samples and ducks  
121 excreted high viral loads for a longer time than chickens, although they did not show  
122 symptoms of disease. Avian wild-type and mutant H7 hemagglutinins bound to both  
123  $\alpha$ 2,3-linked NeuAc and sialyl-LewisX epitopes (an  $\alpha$ 2,3-linked NeuAc presented on an  
124 *N*-acetyllactosamine (LacNAc) with an additional fucose moiety  $\alpha$ 1,3-linked to the *N*-  
125 acetylglucosamine of the LacNAc), but only when presented in complex *N*-glycans.  
126 These findings improve the understanding of equine-specific adaptations in avian H7  
127 receptor binding, viral replication, and pathogenicity while assessing the potential of  
128 interspecies transmission of these viruses.

129

130

## 131 **Results**

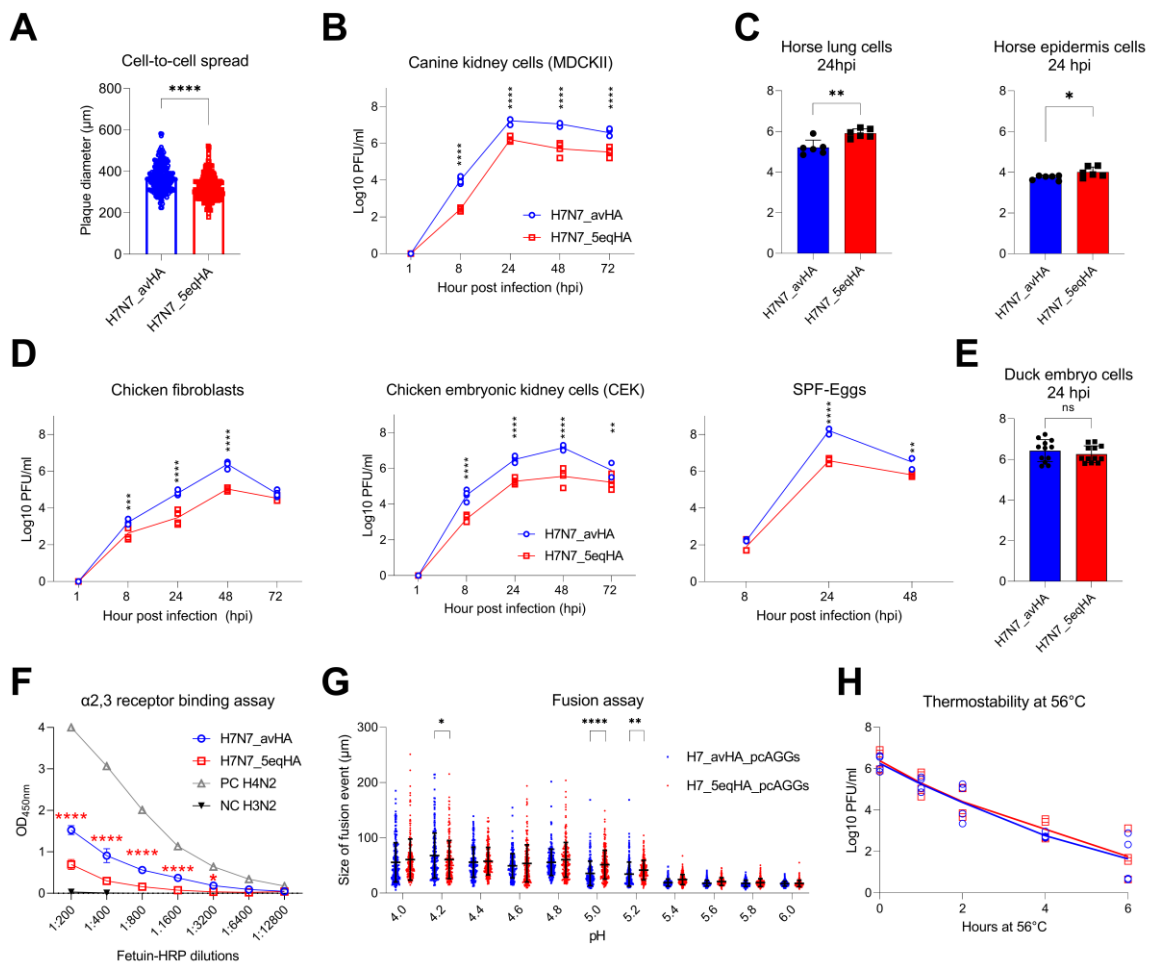
### 132 **NeuGc-specific mutations have differential effects in chicken, duck, and horse** 133 **cells**

134 Previously, we investigated the molecular determinants for binding of avian H7 IAVs  
135 to NeuGc and found that five amino acids that are abundant in equine H7 viruses  
136 (128T, 130V, 135E, 189A, and 193R) were responsible for binding to NeuGc, a  
137 common sialic acid in horses (33). Curiously, we observed that these mutations in the  
138 H7 HA of A/turkey/Italy/214845/02 switched the receptor binding specificity from  $\alpha$ 2,3-  
139 linked NeuAc to  $\alpha$ 2,3-linked NeuGc, but did not cause a loss of binding to chicken  
140 trachea and erythrocytes, which do not contain NeuGc (29, 31). This observation  
141 raised the question of whether the infection capabilities of avian viruses with these  
142 equine NeuGc-specific mutations would be affected.

143  
144 To investigate whether the NeuGc-specific mutations would affect the viral fitness of  
145 an avian virus *in vitro*, we rescued A/chicken/Germany/R28/2003 as a wild-type (WT)  
146 H7N7 HPAIV (designated H7N7\_avHA) and a mutant of this virus carrying the five  
147 NeuGc-specific mutations in the HA (designated H7N7\_5eqHA). The RBS of  
148 H7N7\_avHA is identical to the RBS of A/turkey/Italy/214845/02, which we used in our  
149 previous publication (33). Sequence analysis of avian and equine H7 sequences  
150 showed that all five amino acid residues are highly conserved in equine H7 IAVs (97-  
151 100%), but also naturally occur in some of the analyzed avian H7 HA sequences  
152 (Table S1).

153  
154 The impact of the equine-specific residues on cell-to-cell spread and viral growth  
155 kinetics was investigated in various cell lines (Fig. 1A-E). The viruses' ability to spread  
156 from one cell to another was analyzed in a plaque assay using MDCKII cells, which  
157 are the most commonly used cells for IAV replication assays (34). The NeuGc-specific  
158 residues significantly reduced the intercellular spread at 72 hours post-infection (hpi)  
159 in MDCKII cells (Fig. 1A) and the replication in these cells (Fig. 1B). The NeuGc-  
160 specific mutations significantly increased viral replication in equine lung cells (PLU-R)  
161 and equine epidermal cells (E.Derm) 24 hpi (Fig. 1C). In contrast, H7N7\_5eqHA  
162 replication in chicken fibroblasts, primary CEK cells, and SPF eggs was significantly  
163 reduced compared to WT H7N7\_avHA (Fig. 1D). However, no significant differences

164 between replication of H7N7\_avHA and H7N7\_5eqHA were observed in duck embryo  
 165 fibroblast cells 24 hpi (Fig. 1E). These findings indicate that the equine-specific amino  
 166 acid residues in the RBS of H7 HPAIV reduced cell-to-cell spread and viral replication  
 167 in a host-dependent manner. They increased replication of the avian virus in horse  
 168 cells and reduced replication in chicken cells, but replication in duck cells was  
 169 unaffected.



170  
 171 **Figure 1. *In vitro* characterization of WT (H7N7\_avHA) and mutant (H7N7\_5eqHA)**  
 172 **A/chicken/Germany/R28/2003 H7N7 viruses.** (A) Cell-to-cell spread was investigated by measuring  
 173 the diameter of about 100 plaques in MDCKII cells. (B) Viral replication at indicated time points was  
 174 assessed in MDCKII, (C) horse lung and horse epidermal cells, (D) chicken fibroblasts (DF-1), primary  
 175 chicken cells (CEK), SPF embryonated chicken eggs (ECE), and (E) in duck embryo fibroblast cells.  
 176 (F) The receptor binding affinity to  $\alpha$ 2,3-linked NeuAc was measured using  $\alpha$ 2,3-Sia fetuin substrate.  
 177 Human H3N2 virus (specific for  $\alpha$ 2,6-linked NeuAc) was used as a negative control (NC). An avian  
 178 H4N2 virus (specific for  $\alpha$ 2,3-linked NeuAc) was used as a positive control (PC). Shown are  
 179 representative results calculated as means and standard deviations of three independent experiments,  
 180 each was run in duplicates. (G) pH-dependent activation of HA in a fusion assay was measured after  
 181 transfection of quail cells (QM-9) with pCAGGS protein expression vector containing the HA of

182 H7N7\_avHA or H7N7\_5eqHA. Cells were simultaneously transfected with pCAGGS carrying eGFP to  
183 facilitate the evaluation of the assay. Cell fusion was triggered 24 hpi with PBS of different pH values  
184 for two minutes. The diameter of syncytia was measured using Eclipse Ti-S with software NIS-  
185 Elements, version 4.0; Nikon. (H) The thermostability of viruses was measured in duplicates and  
186 repeated twice. The reduction in virus infectivity at indicated time points was assessed by titration of  
187 heated viruses using a plaque test in MDCKII cells and expressed as plaque-forming units per ml  
188 (Log<sub>10</sub> PFU/ml). All results are expressed as means and standard deviations of at least two  
189 independent experiments run in duplicates. Asterisks indicate statistical significance based on p values:  
190 \* ≤ 0.05, \*\* ≤ 0.01, \*\*\* ≤ 0.001, \*\*\*\* ≤ 0.0001.

### 191 **NeuGc-specific mutations reduced the binding affinity to α2,3-NeuAc without a** 192 **significant impact on the pH-dependent HA activation and thermostability**

193 *In vitro* characterization of generated H7N7 viruses in cell culture revealed that NeuGc-  
194 specific mutations affected viral replication and spread in a host-dependent manner  
195 (Fig. 1A-E). To ascertain whether these mutations have an influence on the viruses'  
196 biological properties and thus on viral replication, the receptor binding properties,  
197 thermostability, and pH activation were tested.

198

199 To assess whether the introduction of the NeuGc-specific mutations in the RBS  
200 changed the binding affinity to α2,3-NeuAc, a solid-phase assay using α2,3-linked  
201 fetuin as a substrate was performed (Fig.1F) as previously described (26, 35). An  
202 avian H4N2 virus was used as a positive control for α2,3-NeuAc binding and a human  
203 H3N2 IAV was used as a negative control. We observed that H7N7\_5eqHA had a  
204 significantly lower binding affinity to α2,3-NeuAc than the WT H7N7\_avHA virus (Fig.  
205 1F), which is in accordance with our previously obtained results (33). In addition to  
206 affecting receptor binding properties, mutations in HA1 may affect pH activation of  
207 hemagglutinin and subsequently affect the replication of viruses such as AIV H5N1  
208 [53], although there is limited knowledge of how it affects AIV H7N7. Therefore, we  
209 assessed the potential influence of the five mutations on the pH-dependent fusion-HA  
210 activation by measuring the diameter of cell-to-cell fusion after transfection of avian  
211 cells (QM9) with protein expression vectors (pCAGGS) carrying HA from H7N7\_avHA  
212 (H7\_avHA\_pcAGGs) or H7N7\_5eqHA (H7\_5eqHA\_pcAGGs) (Fig. 1G). Both  
213 hemagglutinins were activated at a broad range of pH values from 4.0 to 6.0. However,  
214 the fusion efficiency of H7\_5eqHA\_pcAGGs in QM9 cells at a pH of 5.0 and 5.2 was  
215 significantly higher than that of H7\_avHA\_pcAGGs. Interestingly, the pH-dependent  
216 activation of H7\_avHA\_pcAGGs was found to be significantly higher at a pH value of



217 4.2 than H7\_5eqHA\_pcAGGs. The size of fusion events at other pH values was  
218 comparable (Fig. 1G).

219

220 The thermostability of the HA is known to be linked to virulence in different influenza  
221 strains (36). Therefore, we evaluated the thermostability of the two viruses at 56°C for  
222 2, 4, and 6 hours, a standard treatment for enveloped viruses (37). Both viruses lost  
223 infectivity at comparable levels indicating that the introduction of equine-specific amino  
224 acids did not affect the thermostability of the HPAIV (Fig. 1H). In conclusion, the  
225 NeuGc-specific mutations reduced the replication of this H7N7 HPAIV in the chicken  
226 cells probably due to reduced binding affinity to the 2,3-NeuAc without significantly  
227 impacting the HA pH-dependent activation and thermostability of the viruses.

228

### 229 **The NeuGc-specific residues significantly reduced virulence in infected** 230 **chickens, but had no impact on virus virulence in ducks**

231 Since the NeuGc-specific mutations had an impact on receptor binding, cell-to-cell  
232 transmission, and viral replication in chicken, but not duck cells (Fig. 1), these  
233 mutations potentially also affect the viruses *in vivo*. Therefore, we performed an animal  
234 experiment in chickens, from which the H7N7\_avHA was originally isolated and which  
235 are economically crucial hosts, and ducks as the natural reservoir species of AIVs.

236

237 Nine SPF chickens and ten Pekin ducks were infected by intravenous (IV) or  
238 intramuscular (IM) injection, respectively with H7N7\_avHA or H7N7\_5eqHA to assess  
239 the viral pathogenicity index (PI) according to the WOAHA standard (38). All ducks  
240 infected IM with H7N7\_avHA or H7N7\_5eqHA survived the animal experiment and  
241 showed no clinical disorders (Table 1). Nevertheless, all ducks seroconverted  
242 indicating a successful infection. The intramuscular pathogenicity index (IMPI) for  
243 H7N7\_avHA and H7N7\_5eqHA in ducks was determined to be 0.0. Conversely,  
244 chickens that were IV-infected with H7N7\_avHA died within a mean time of death  
245 (MDT) of 4.6 days post-infection (dpi). All chickens displayed clinical signs of infection  
246 and an intravenous pathogenicity index (IVPI) of 2.4 was calculated. H7N7\_avHA is  
247 therefore classified as an HPAIV according to the WOAHA classification (IVPI > 1.2  
248 indicates an AIV as HPAIV). Interestingly, the introduction of the five equine mutations  
249 into the avian HA reduced mortality to 3 out of 9 IV-infected chickens with  
250 H7N7\_5eqHA, but all chickens exhibited transient mild to moderate clinical signs.

251 Notably, IV-infected chickens with H7N7\_5eqHA died earlier compared to those IV-  
 252 infected with H7N7\_avHA, with an MDT of 3.0 days (Table 1), although the differences  
 253 in MDT of both groups were not statistically significant. Nevertheless, the IVPI of  
 254 H7N7\_5eqHA inoculated chickens was determined to be 1.5, which is still classified  
 255 as HPAIV. All remaining chickens subsequently developed antibodies.

256

257 **Table 1. The pathogenicity indices of H7N7 viruses after the injection of chickens and ducks.**

258 The table shows the mortality, morbidity, and seroconversion of intravenous infected (IV) chickens and  
 259 intramuscular infected (IM) Pekin ducks. In addition, the calculated mean time of death (MDT) in days  
 260 post-infection (dpi) and intravenous (IVPI) or intramuscular (IMPI) pathogenicity indices are shown.

Virus	Animal	Group	Mortality	Mean time of death (MDT)	Morbidity	Pathogenicity index (PI)	Seropositive birds/total birds
<b>H7N7_avHA</b>	Chickens	IV infected	9/9	4.6	9/9	IVPI: 2.4	n.a
	Ducks	IM infected	0/10	n.a. *	0/10	IMPI: 0.0	10/10
<b>H7N7_5eqHA</b>	Chickens	IV infected	3/10	3.0	10/10	IVPI: 1.5	7/7
	Ducks	IM infected	0/10	n.a. *	0/10	IMPI: 0.0	10/10

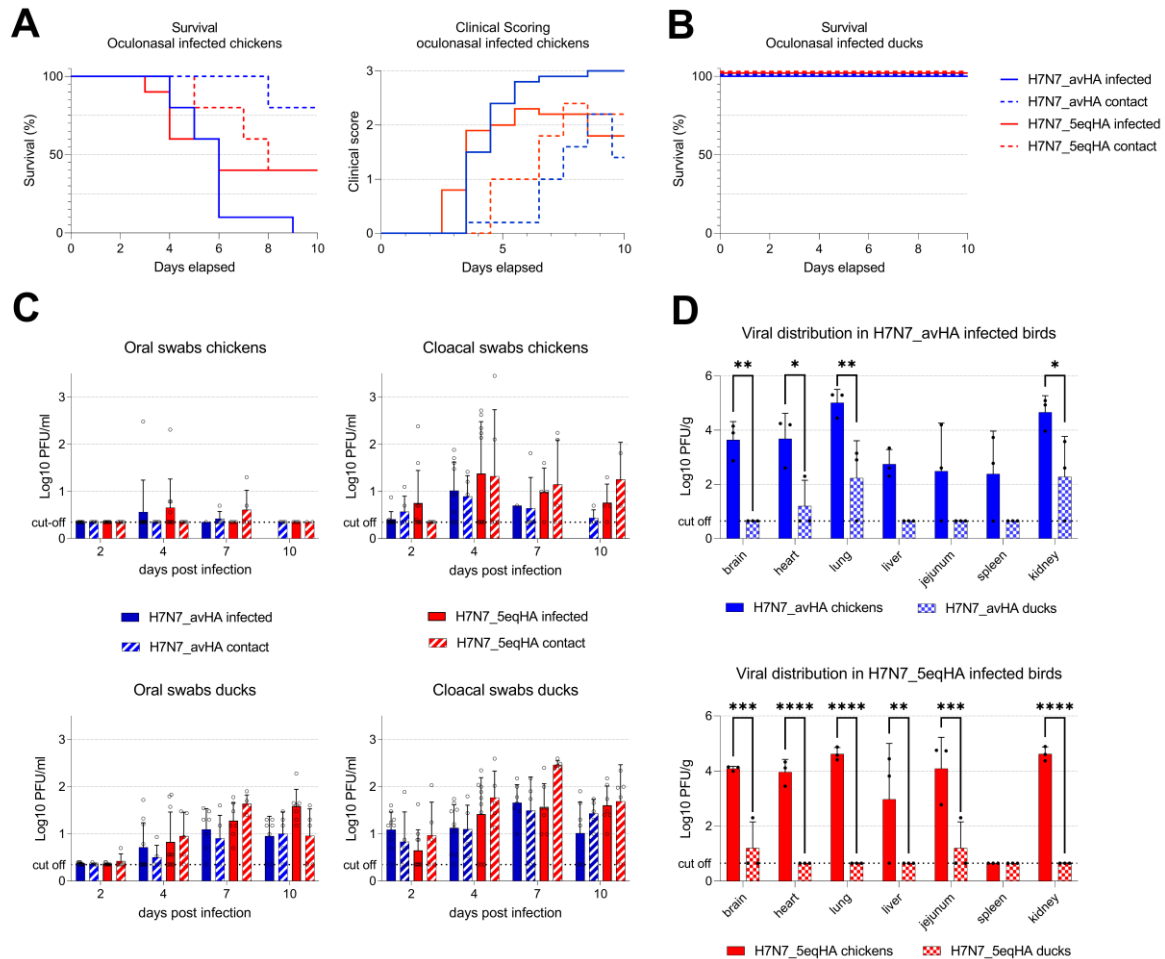
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\*n.a. = not applicable

263 In the second animal experiment, we wanted to mimic the natural course of infection.  
 264 Therefore, ten chickens and ten ducks were inoculated by the oculonasal (ON) route.  
 265 Furthermore, at 1 dpi five chickens and five ducks were added to each group to assess  
 266 chicken-to-chicken or duck-to-duck transmission. All chickens primarily inoculated with  
 267 H7N7\_avHA died within an MDT of 5.7 dpi, with an average CS of 1.7 (Fig. 2A, Table  
 268 2). Only one contact chicken died on the eighth dpi in the avian H7N7 (H7N7\_avHA)  
 269 infected group. However, all contact chickens displayed signs of morbidity (Table 2).  
 270 Six out of ten chickens infected with H7N7\_5eqHA died with an MDT of 4.5 days. In  
 271 this group, three out of five contact chickens died with MDT of 7.3 days, and the two  
 272 remaining chickens showed transient mild to moderate clinical signs. The average PI  
 273 was 1.4 for the primarily inoculated chickens (Fig. 2A, Table 2). Conversely, and  
 274 similar to the IM-injected ducks, neither the ON-inoculated nor the contact ducks in  
 275 either group displayed any signs of illness. All ducks survived until the end of the  
 276 animal trial (Table 2, Fig. 2B). Seropositive results were recorded for all remaining  
 277 chickens and ducks using an anti-NP ELISA (Table 2). These findings confirm the

278 results from the IVPI and IMPI-infected birds, as H7N7\_5eqHA is less lethal in  
 279 chickens than H7N7\_avHA and ducks are not affected at all. Taken together, and  
 280 regardless of the infection routes, the equine-adaptive mutations reduced HPAIV  
 281 H7N7 virulence in chickens, while ducks were clinically resistant to both viruses.



282

283 **Figure 2. *In vivo* characterization of WT (H7N7\_avHA) and mutant (H7N7\_5eqHA)**  
 284 **A/chicken/Germany/R28/2003 H7N7 virus.** (A) Survival and clinical score of ON infected chickens  
 285 throughout the animal experiment. (B) Survival curve of ON infected Pekin ducks. (C) Analysis of oral  
 286 and cloacal swab samples taken from chickens and ducks in plaque tests expressed as Log<sub>10</sub> PFU/ml.  
 287 (D) The viral distribution in duck and chicken organs was analyzed in plaque tests and expressed as  
 288 PFU/gram. Asterisks indicate statistical significance based on p values \* ≤ 0.05, \*\* ≤ 0.01, \*\*\* ≤ 0.001,  
 289 \*\*\*\* ≤ 0.0001. ns = not significant. Dashed lines indicate the predicted detection limit of the plaque  
 290 assay (cut-off).

291

292

293 **Table 2. *In vivo* data from oculonasal infected chickens and Pekin ducks.** The table shows the  
 294 sick (morbidity) and dead (mortality) animals per group, as well as the results from sera analysis in a  
 295 competitive NP ELISA. Calculated mean time to death (MDT) in days post-infection as well as the  
 296 clinical score (CS) are shown.

Virus	Animal	Group	Mortality	Mean time of death (MDT)	Morbidity	Clinical Score (CS)	Seropositive birds /total birds
<b>H7N7_ avHA</b>	Chickens	ON infected	10/10	5.7	10/10	1.7	n.a
		ON Contact	1/5	8.0	5/5	0.6	4/5
	Ducks	ON infected	0/10	n.a.*	0/10	0.0	7/7
		ON Contact	0/5	n.a.*	0/5	0.0	5/5
<b>H7N7_ 5eqHA</b>	Chickens	ON infected	6/10	4.5	10/10	1.4	4/4
		ON Contact	3/5	7.3	5/5	1.0	2/2
	Ducks	ON infected	0/10	n.a.*	0/10	0.0	7/7
		ON Contact	0/5	n.a.*	0/5	0.0	5/5

\*n.a. = not applicable

297  
298

299 **The NeuGc-specific residues did not affect virus replication or excretion in**  
 300 **chickens, but ducks are potentially silent spreaders of H7N7 viruses**

301 We further determined the effect of the five equine mutations on viral loads in swab  
 302 and organ samples obtained from ON-inoculated birds and their contacts. Oral and  
 303 cloacal swabs collected 2, 4, 7, and 10 dpi were tested using plaque assay. In chickens  
 304 and ducks, the level of virus shedding from the cloacal route was higher compared to  
 305 the oral route, although the differences were not statistically significant (Fig. 2C).  
 306 Interestingly, ducks excreted high viral loads by the oral and fecal routes for a longer  
 307 time than chickens. Moreover, the viral distribution in different organs (brain, heart,  
 308 lungs, liver, jejunum, spleen, and kidneys) obtained 4 dpi from three birds of each  
 309 group was broader in chickens than in ducks. In H7N7\_avHA-infected chickens, the  
 310 viral load was significantly higher in the brain, heart, liver, and kidneys compared to

311 infected ducks (Fig. 2D). Similarly, in H7N7\_5eqHA-infected chickens, the viral load  
312 was significantly higher in all organs except the spleen compared to ducks.

313

314 In conclusion, we observed increased levels of viral shedding via the cloacal routes  
315 and prolonged shedding of viruses in ducks compared to chickens. The viral  
316 distribution in chicken organs was broader than in ducks, which may explain the high  
317 mortality in chickens. These findings raise concerns about the potential spread of  
318 H7N7 viruses, particularly by ducks as silent spreaders. The high and prolonged  
319 shedding of the H7 viruses in ducks, even without exhibiting clinical symptoms, pose  
320 a potential risk for their reintroduction to hosts that have a high presence of NeuGc,  
321 like pigs and horses.

322

### 323 **Avian H7 IAVs bind both $\alpha$ 2,3-linked NeuAc and sialyl-LewisX epitopes**

324 The animal experiments showed that both chickens and ducks were infected by the  
325 NeuGc-specific H7 viruses (Fig. 2), although both species are known to not express  
326 NeuGc (29, 31). This strongly suggests that in our previous research, in which we  
327 observed NeuGc-specificity of this mutant (S128T, I130V, A135E, T189A, and K193R)  
328 H7 HA (33), we overlooked the binding to one of the many other glycans that may be  
329 present in nature. We hypothesized that sialyl-LewisX (sLe<sup>x</sup>) epitopes are important  
330 as they have recently been shown to be involved in H7 IAV infection (39, 40) and are  
331 bound by nearly all IAV subtypes (41-49). The sLe<sup>x</sup> epitope consists of an  $\alpha$ 2,3-linked  
332 NeuAc presented on an *N*-acetyllactosamine (LacNAc) with an additional fucose  
333 moiety  $\alpha$ 1,3-linked to the *N*-acetylglucosamine (GlcNAc) of the LacNAc. The sLe<sup>x</sup>  
334 epitopes are present in some species and tissues, such as chicken trachea and colon,  
335 guinea fowl trachea, turkey respiratory tract, and human lung (45, 50-55). Most  
336 research investigating binding to the sLe<sup>x</sup> epitope has been performed using a  
337 tetrasaccharide sLe<sup>x</sup> epitope, due to a lack of biologically relevant glycans.

338

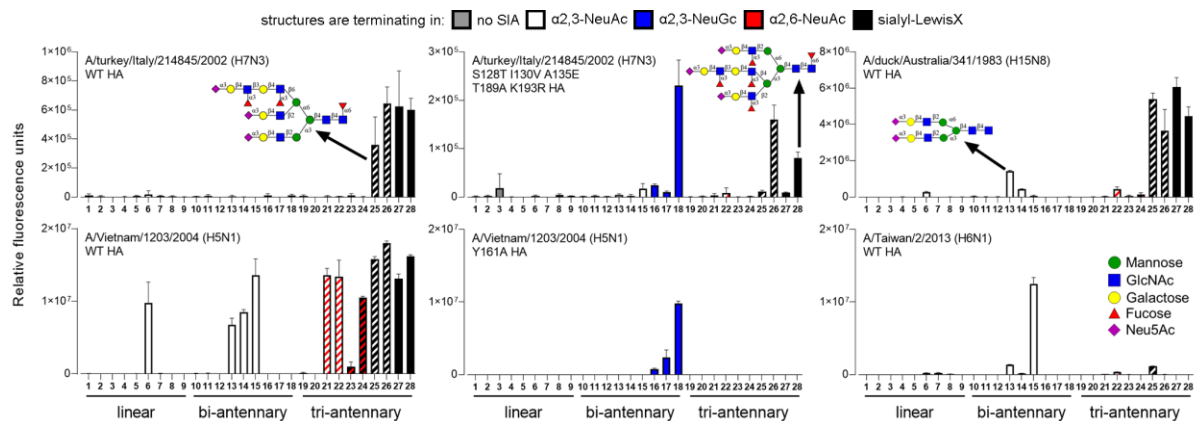
339 Here, we investigated which exact glycans are bound by WT and mutant avian H7  
340 HAs to explain how chickens and ducks are infected by NeuGc-specific H7 viruses.  
341 Since the complex glycan structure can influence receptor binding (14, 22-25), we  
342 here focused on biologically relevant complex *N*-glycans presenting  $\alpha$ 2,3-linked  
343 NeuAc,  $\alpha$ 2,3-linked NeuGc,  $\alpha$ 2,6-linked NeuAc, and sLe<sup>x</sup> epitopes (Fig. S1). These

344 glycans were printed on glass slides and after incubation of the slides with the HAs  
 345 and fluorescent secondary antibodies, the glycan-HA binding was evaluated.

346

347 Whereas the WT HA of A/turkey/Italy/214845/02 (H7tu) previously only showed  
 348 binding to glycans terminating in  $\alpha$ 2,3-linked NeuAc (33), here we observed a strong  
 349 preference for tri-antennary *N*-glycans presenting at least one sLe<sup>x</sup> epitope (glycans  
 350 **25-28**, Fig. 3). Both glycans solely presenting sLe<sup>x</sup> epitopes (**27**, **28**) and glycans  
 351 presenting a sLe<sup>x</sup> on one arm and  $\alpha$ 2,3-linked NeuAc on the other two arms (**25**, **26**)  
 352 were bound. For the latter, it cannot be distinguished whether binding is caused by the  
 353 sLe<sup>x</sup> or the  $\alpha$ 2,3-linked NeuAc. Interestingly, binding to sLe<sup>x</sup> epitopes presented on  
 354 linear glycans (**7-9**) was not observed. Furthermore, steric hindrance due to the  
 355 presence of  $\alpha$ 2,6-linked NeuAc on two arms, besides the sLe<sup>x</sup> on one arm, appeared  
 356 to be present, as glycan **23** and **24** were not bound. We also showed that the mutant  
 357 H7tu HA bound both glycans terminating in  $\alpha$ 2,3-linked NeuGc, as well as sLe<sup>x</sup>-  
 358 presenting tri-antennary *N*-glycans (Fig. 3). In conclusion, the observed binding to  
 359 sLe<sup>x</sup> (Fig. 3) showed that the previously studied mutant avian H7 HAs were not strictly  
 360 specific for NeuGc and may explain why these HAs bound to chicken tissue and  
 361 erythrocytes previously (33). This sLe<sup>x</sup>-binding possibly also explains why ducks and  
 362 chickens could be infected by the NeuGc-adapted avian H7 virus (Fig. 2).

363



364

365 **Figure 3. Avian H7 HAs bind to sialyl-LewisX epitopes.** Synthetic glycans were used to assess the  
 366 receptor binding of the IAV HAs (A/turkey/Italy/214845/2002 H7, A/duck/Australia/341/1983 H15,  
 367 A/Vietnam/1203/2004 H5, and A/Taiwan/2/2013 H6). The glycans were terminating in galactose (no  
 368 SIA, grey),  $\alpha$ 2,3-linked NeuAc (white),  $\alpha$ 2,3-linked NeuGc (blue),  $\alpha$ 2,6-linked NeuAc (red), or sialyl-  
 369 LewisX (black). Bars with two colors indicate glycans terminating in different epitopes on different arms.  
 370 Fig. S1 presents all structures that are present on the array. Bars represent the mean  $\pm$  SD (n=4).

371 Furthermore, we examined the receptor specificity of the H15 HA of  
372 A/duck/Australia/341/1983, the closest related subtype to H7. The H15 HA showed a  
373 similar binding phenotype to the H7tu HA and bound  $\alpha$ 2,3-linked NeuAc and sLe<sup>X</sup>,  
374 while structures presenting  $\alpha$ 2,6-linked NeuAc on the other arms were not bound (Fig.  
375 3). Interestingly, H15 HAs were previously not known to bind to sLe<sup>X</sup> epitopes. The  
376 steric hindrance due to  $\alpha$ 2,6-linked NeuAc appeared to be specific for the H7 and H15  
377 HAs, since the WT H5 HA from A/Vietnam/1203/2004 (H5VN) showed binding to all  
378 tri-antennary *N*-glycans presenting at least one sLe<sup>X</sup> epitope, regardless of the  
379 terminal epitopes presented on the other arms (Fig. 3). Consistent with the results  
380 from H7 and H15 HAs, the WT H5VN HA only bound to sLe<sup>X</sup> epitopes when presented  
381 on tri-antennary *N*-glycans, and not linear glycans, possibly due to a multivalency  
382 effect because of the high density of binding epitopes in one glycan. Furthermore, an  
383 HA that we previously used as a control for specific binding of NeuGc (the Y161A HA  
384 mutant A/Vietnam/1203/2004 H5N1) bound specifically to NeuGc and not sLe<sup>X</sup> (Fig.  
385 3). Interestingly, the H6 HA from A/Taiwan/2/2013 was strictly specific for  $\alpha$ 2,3-linked  
386 NeuAc and did not bind to glycans that present other epitopes on the other arms (**25**  
387 and **26**). The results showed that the fine receptor specificity is highly dependent on  
388 the IAV and the exact complex glycan structure.

389

390 Nevertheless, the binding of the H7 HAs to sLe<sup>X</sup> epitopes does not explain the infection  
391 in ducks, since ducks are generally assumed not to present NeuGc nor sLe<sup>X</sup>  
392 epitopes (29, 31, 50). A tissue stain using anti-sialyl-LewisX antibodies revealed that,  
393 indeed, no sLe<sup>X</sup> epitopes were found on duck colon and tracheal tissues (Fig. 4A).  
394 Therefore, we aimed to investigate whether the binding of these avian H7 HAs was  
395 truly dependent on sLe<sup>X</sup> epitopes. We performed assays using a fucosidase  
396 E1\_10125 from *Ruminococcus gnavus* E1, which specifically cleaves the fucose  
397 moiety from the sLe<sup>X</sup> epitope (56) (Fig. 4B). The anti-sialyl-LewisX antibody showed  
398 that no more sLe<sup>X</sup> epitopes remained on the chicken trachea after fucosidase  
399 treatment. However, the binding of both WT and mutant H7tu (Fig. 4B) HA to chicken  
400 trachea remains unchanged after fucosidase treatment, indicating that  $\alpha$ 2,3-linked  
401 NeuAc is bound in a tissue section.

402

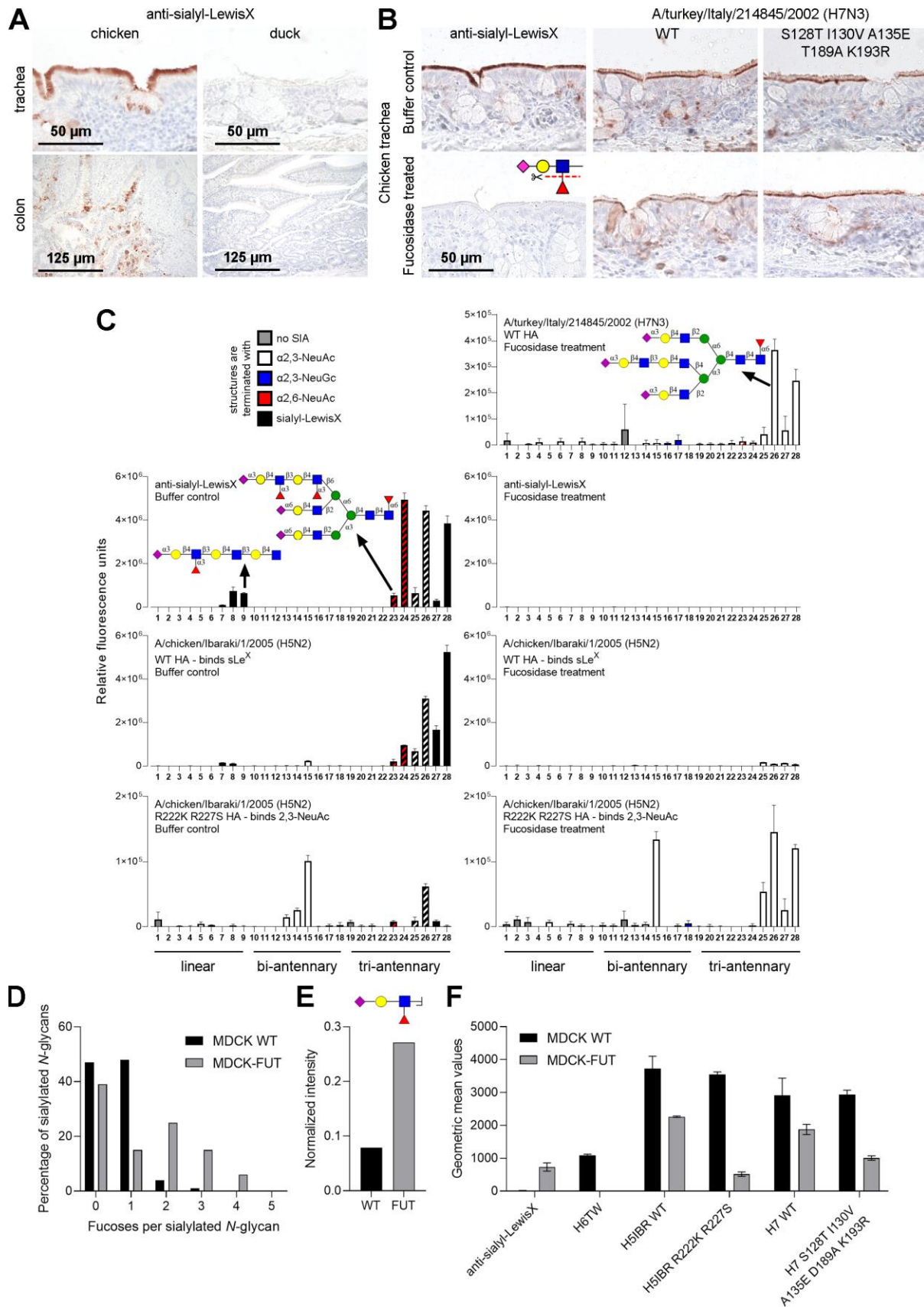
403 To investigate which exact glycans may have been involved in this binding to the  
404 chicken trachea, we treated the glycan microarray with fucosidase (Fig. 4C). Using an

405 anti-sLe<sup>x</sup> antibody that bound all sLe<sup>x</sup>-containing structures on the glycan microarray  
406 as a control, we indeed showed that all sLe<sup>x</sup> epitopes were removed after fucosidase  
407 treatment of the array. This suggests that no sLe<sup>x</sup> epitopes remained on the chicken  
408 trachea either after fucosidase treatment (Fig. 4B). As additional controls, we used the  
409 WT and R222K R227S mutant H5 HAs of A/chicken/Ibaraki/1/2005 (H5IBR), which  
410 are, respectively, specifically binding to sLe<sup>x</sup> structures and  $\alpha$ 2,3-linked NeuAc (43).  
411 After fucosidase treatment, these WT and mutant H5IBR HA controls showed,  
412 respectively, no binding to sLe<sup>x</sup> glycans and increased binding to sLe<sup>x</sup> glycans (that  
413 are now converted to  $\alpha$ 2,3-linked NeuAc) (Fig. 4C). Interestingly, the H7tu WT HA still  
414 bound to structures **25** to **28** (of which the sLe<sup>x</sup> is converted to  $\alpha$ 2,3-linked NeuAc)  
415 after fucosidase treatment, but not bi-antennary *N*-glycans presenting  $\alpha$ 2,3-linked  
416 NeuAc (**13-15**, Fig. S1, Fig. 4C), suggesting that tri-antennary *N*-glycans are preferred  
417 as receptors for H7tu. In conclusion, both  $\alpha$ 2,3-linked NeuAc and sLe<sup>x</sup> epitopes  
418 presented on the tri-antennary *N*-glycans are bound efficiently by the H7tu WT HA.

419  
420 To further investigate binding to sLe<sup>x</sup> glycans, we used MDCK WT and MDCK-FUT  
421 cells. The latter overexpress the chicken fucosyltransferase genes *FUT3*, *FUT5*, and  
422 *FUT6* (50), which was expected to increase the amount of sLe<sup>x</sup> epitopes that are  
423 presented on the cells. To investigate whether indeed increased amounts of sLe<sup>x</sup>  
424 epitopes were presented on the cells, we employed mass spectrometry (MS) methods.  
425 We first investigated the released *N*-glycans from MDCK WT and FUT cells by HILIC-  
426 IMS-QTOF positive mode MS and found that MDCK-FUT cells presented a higher  
427 number of fucoses on sialylated *N*-glycans than MDCK WT cells (Fig. 4D, Table S2).  
428 To further investigate whether the fucoses were present in sLe<sup>x</sup> epitopes, we analyzed  
429 the *N*-glycans using fragmentation in LC-MS/MS, which indeed showed a higher  
430 relative abundance of sLe<sup>x</sup> fragments (oxonium ions of  $m/z$  803.2928) on the MDCK-  
431 FUT cells (Fig. 4E). We then continued to use these MDCK WT and FUT cells in flow  
432 cytometry analysis. The controls (anti-sLe<sup>x</sup> and the HA of A/Taiwan/2/2013 H6N1,  
433 which is specific for  $\alpha$ 2,3-linked NeuAc (Fig. 3)) showed that the amount of sLe<sup>x</sup> on  
434 MDCK WT cells and the amount of  $\alpha$ 2,3-linked NeuAc on MDCK-FUT cells was very  
435 low (Fig. 4F). Surprisingly, the H5IBR HAs (WT and mutant) that were assumed to be  
436 specific for sLe<sup>x</sup> and  $\alpha$ 2,3-linked NeuAc, respectively, bound well to both cell types.  
437 Similar to the result in the glycan microarray and tissue stains, the H7 (and H15) WT  
438 and mutant HAs bound to both cell types. In conclusion, both  $\alpha$ 2,3-linked NeuAc and



439 sLe<sup>x</sup> epitopes appear to be bound efficiently by the H7tu, but binding depends on the  
 440 exact glycan structure.



441

442 **Figure 4. Avian H7 HAs bind both  $\alpha$ 2,3-linked NeuAc and sialyl-LewisX epitopes.** (A) The presence  
443 of sialyl-LewisX epitopes on chicken and duck trachea and colon was investigated using anti-sialyl-  
444 LewisX antibodies. (B) The binding of anti-sialyl-LewisX antibodies and H7 HAs to chicken tracheal  
445 tissue (with and without fucosidase treatment) was assessed. Tissue binding was visualized using AEC  
446 staining. (C) Synthetic glycans (with and without fucosidase treatment) were used to assess the binding  
447 of the anti-sialyl-LewisX antibody, the WT H7 HA of A/turkey/Italy/214845/02, and H5 HAs of  
448 A/chicken/Ibaraki/1/2005. (D) The *N*-glycans of MDCK WT and MDCK-FUT cells were investigated  
449 using HILIC-IMS-QTOF positive mode mass spectrometry. The number of fucoses per sialylated *N*-  
450 glycan was analyzed for both cell types. Further analysis is presented in Table S2. (E) The *N*-glycans  
451 were further analyzed using LC-MS/MS. The oxonium ions of  $m/z$  803.2928 (sLe<sup>X</sup>) were identified and  
452 normalized to the core fragments. Mean and standard errors (n=3) are shown. (F) Flow cytometry  
453 measurements were performed to assess the binding anti-sialyl-LewisX antibodies and HAs to MDCK  
454 WT and MDCK-FUT cells. Triplicate measurements were performed, of which the mean and standard  
455 deviation are displayed.

## 456 Discussion

457 Here, we studied the effects of equine NeuGc-adapting mutations (S128T, I130V,  
458 A135E, T189A, and K193R) in avian H7 IAVs *in vitro* and *in vivo*. These viruses are  
459 potentially candidates for interspecies transmission between avian and mammalian  
460 species expressing NeuGc receptors. The insertion of equine NeuGc-adapting  
461 mutations resulted in stable and viable viruses and increased viral replication in horse  
462 cells. While the mutations reduced viral replication in chicken and dog cells,  
463 interestingly the replication in duck cells was not affected. *In vivo*, the NeuGc-adapting  
464 mutations not only reduced the pathogenicity index in intravenously infected chickens  
465 but also mortality and morbidity in oculonasal-infected chickens. In ducks, on the other  
466 hand, neither virus caused signs of illness or increased mortality. Nevertheless, ducks  
467 shed high amounts of virus for a longer time compared to chickens. Here, NeuGc-  
468 adapting mutations were not disadvantageous in viral shedding compared to the WT  
469 HPAIV. The NeuGc-adapted H7 was additionally found to bind  $\alpha$ 2,3-linked NeuAc and  
470 sialyl-LewisX (sLe<sup>X</sup>) epitopes, but only when these epitopes were presented on tri-  
471 antennary *N*-glycans. Binding to these epitopes explains why ducks and chickens  
472 could be infected and emphasizes the risk of interspecies transmission of H7 IAVs.

473

474 Although sLe<sup>X</sup> epitopes were identified as potential receptors for the studied H7  
475 viruses, it is currently unclear whether sLe<sup>X</sup> is used in IAV infections as a functional  
476 receptor, or whether it has other functions. It has been suggested that the presence of

477 sLe<sup>x</sup> facilitates H7 IAV infection . If sLe<sup>x</sup> binding is important in IAV infection, this may  
478 cause a species barrier or act as an intermediate receptor since some species and  
479 tissues, such as the chicken trachea and colon, guinea fowl trachea, turkey respiratory  
480 tract, and human lung present sLe<sup>x</sup> epitopes (45, 50-55).

481

482 The molecular determinants for the binding of H7 viruses to sLe<sup>x</sup> epitopes are currently  
483 unknown. Since we showed that not all IAVs bind to sLe<sup>x</sup> epitopes, such as the H6  
484 HA of A/Taiwan/2/2013 (Fig. 3), likely certain amino acids are responsible for the  
485 binding to sLe<sup>x</sup>. For H5 viruses, it was shown that mutations K222R/Q and S227R in  
486 the HA convert from binding to  $\alpha$ 2,3-linked NeuAc to sLe<sup>x</sup> (43, 49, 51). Especially, the  
487 lysine at position 222 was shown to sterically hinder binding to sLe<sup>x</sup> epitopes (57),  
488 while a glutamine or arginine at that position enables, potentially through a hydrogen  
489 bond, sLe<sup>x</sup> binding. Indeed, the H7 viruses that were investigated here contain a  
490 glutamine (Q) at position 222, which is highly conserved in H7 viruses (51) and partially  
491 explains the binding to sLe<sup>x</sup>. However, position 227 is also a glutamine in the  
492 investigated H7 viruses, of which the effect on sLe<sup>x</sup> binding is currently unknown.  
493 Elsewhere, the presence of a lysine at position 193 is reported to be important for sLe<sup>x</sup>  
494 binding and, indeed, the H7tu contains a 193K (40). Additionally, amino acids at other  
495 positions, which have not been investigated yet, may also affect the binding to sLe<sup>x</sup>  
496 epitopes.

497

498 Previously, IAVs from all subtypes, except H15, were shown to bind sLe<sup>x</sup> epitopes  
499 (41-49). Using tri-antennary *N*-glycans presenting sLe<sup>x</sup> epitopes, we here showed that  
500 also H15 IAVs, the closest related subtype to H7, are capable of binding sLe<sup>x</sup> epitopes.  
501 Furthermore, the investigated avian H7 HAs were previously not known to bind to sLe<sup>x</sup>  
502 epitopes, as binding was only observed when the epitopes were presented on tri-  
503 antennary *N*-glycans and not linear glycans. Additionally, the WT H7 HA appeared to  
504 bind stronger to  $\alpha$ 2,3-linked NeuAc when presented on tri-antennary *N*-glycans (sLe<sup>x</sup>  
505 after fucosidase treatment) than bi-antennary *N*-glycans or linear glycans. The H7tu  
506 also appeared to bind stronger to tri-antennary *N*-glycans presenting the  $\alpha$ 2,3-linked  
507 NeuAc on an elongated MGAT4 arm (**26** and **28**) instead of an elongated MGAT5 arm  
508 (**25** and **27**) (Fig. 4C, Fig. S1), although this was not consistent throughout the  
509 replicates. Using these sLe<sup>x</sup>-presenting *N*-glycans in combination with other HAs may  
510 reveal the binding of more IAVs to sLe<sup>x</sup> and the role of these epitopes in IAV infection.

511 These observations highlight the relevance of looking beyond the terminal epitope and  
512 considering the fine receptor specificity when investigating IAV receptor binding.

513

514 The distribution and types of Sias are species-specific and variable throughout the  
515 respiratory tract of IAV-susceptible species (17). HA specificity is often adapted to the  
516 particular Sia receptors present in the host (14). Thus, interspecies transmission and  
517 establishment in a new host requires a successful adaptation of HA binding specificity  
518 to the new host environment as seen in equine H7N7 viruses originating from avian  
519 H7 viruses (10). Although equine H7N7 IAVs are thought to be extinct (12, 13), the  
520 amino acid residues coding for the NeuGc binding specificity persist in avian H7  
521 sequences (Table S1), enabling a potential re-emergence of NeuGc-binding viruses.  
522 The avian H7 IAVs with equine-adapted mutations that we investigated not only bound  
523 to equine-specific NeuGc-receptors but were also able to replicate and infect avian  
524 hosts (Fig. 2). The viruses with equine-adapted NeuGc-specific mutations may not be  
525 as effective in avian  $\alpha$ 2,3-linked NeuAc receptor binding and viral replication as WT  
526 avian virus but still show infection *in vivo*. Furthermore, reassortant viruses with an  
527 equine H7N7 HA and other genes from a chicken H5N2 IAV were shown to be lethal  
528 in chickens (11). This suggests a potential for transmission of equine-adapted viruses  
529 with NeuGc binding specificity back to avian species like chickens or ducks, for  
530 example, due to the close proximity of these species in farms. Our observations  
531 highlight the relevance of considering the fine receptor binding specificity when  
532 investigating the effect of species-specific adaptations in the RBS of HA and their  
533 potential in interspecies transmission events.

534

## 535 **Materials & Methods**

### 536 **Cell culturing and preparation of cell lysates**

537 The Biobank of the Friedrich-Loeffler-Institut (FLI), Greifswald Insel-Riems, Germany  
538 provided the following cell cultures for the *in vitro* characterization of the viruses:  
539 human embryonic kidney cells (HEK293T), Douglas Foster-1 cells (DF-1), Madin-  
540 Darby Canine Kidney type II cells (MDCKII), quail muscle 9 cells (QM-9), horse  
541 epidermal cells (E.Derm), horse lung cells (PLU-R), and duck embryo cells (SEF-R).  
542 In addition, 11d-old specific-pathogen-free embryonated chicken eggs SPF-ECE (Valo

543 BioMedia, Germany) and chicken embryonic kidney cells (CEK) isolated from 18d-old  
544 SPF-ECE were used to perform replication kinetics (58).

545

546 MDCKII and PLU-R cells were cultured in minimal essential medium (MEM) with 10%  
547 fetal calf serum (FCS) containing Hank's, Earls salts, NaHCO<sub>3</sub>, sodium pyruvate, and  
548 non-essential amino acids. For HEK293T, DF-1, QM-9, and SEF-R Iscove's Modified  
549 Dulbecco's medium (IMDM) with 10% FCS, Ham's F12 nutrient mix, and glutamine  
550 was used. E.Derm and CEK cells were cultured in Eagle's MEM and different  
551 concentrations of NaHCO<sub>3</sub>. HEK293S GnTI(-) cells were cultured in DMEM with 10%  
552 FCS. All cells were cultured at 37°C with 5% CO<sub>2</sub>.

553

554 MDCK WT (CCL-34) and MDCK-FUT (50) (a kind gift from Takahiro Hiono) cells were  
555 cultured in DMEM (Gibco) with 10% FCS (S7524, Sigma) and 1% penicillin and  
556 streptomycin (Sigma). MDCK-FUT cells were maintained with an additional 500 µg/ml  
557 G418 sulfate. MDCK-FUT cells overexpress the chicken fucosyltransferase genes  
558 *FUT3*, *FUT5*, and *FUT6* (50). Cells were cultured at 37°C with 5% CO<sub>2</sub>. Detaching of  
559 the cells was always done using 1X TrypLE Express Enzyme (12605010, Thermo  
560 Fisher Scientific), using 2 ml in a T75 flask, at a confluency of approximately 90%. Cell  
561 lysates were obtained using TrypLE Express Enzyme and RIPA lysis buffer as  
562 described previously (59).

563

## 564 **Viruses**

565 The influenza viruses were obtained from different cooperation partners:  
566 A/chicken/Germany/R28/2003 (H7N7) (designated H7N7\_avHA) was provided by  
567 Timm C. Harder, head of the reference laboratory for avian influenza virus, Friedrich-  
568 Loeffler-Institut (FLI), Greifswald Insel-Riems, Germany. AIV  
569 A/quail/California/D113023808/2012 (H4N2) was supplied by Beate Crossley from the  
570 California Animal Health and Food Safety Laboratory System, Department of Medicine  
571 and Epidemiology, University of California, Davis, United States. Stephan Pleschka  
572 and Ahmed Mostafa from Justus-Liebig-University, Gießen, Germany provided the  
573 human isolate A/Victoria/1975 (H3N2).

574

575 The avian sequence containing the five equine mutations S128T, I130V, A135E,  
576 T189A, and K193R (H3 numbering) was ordered from GenScript and inserted into the  
577 HA of A/chicken/Germany/R28/2003 in a pHWSccdB vector by restriction enzyme  
578 cloning. The IAV carrying these 5 mutations (designated H7N7\_5eqHA) was  
579 generated in the backbone A/chicken/Germany/R28/2003 using reverse genetics. The  
580 virus was rescued in HEK293T cells, propagated in 9- to 11-d-old SPF eggs, and  
581 pooled for further use. Sequence analysis of different isolated viral pools revealed a  
582 stable establishment of the five mutations in the HA without a reversion to the WT  
583 sequence.

584

### 585 **$\alpha$ 2,3-linked NeuAc receptor binding affinity assay**

586 The binding of H7N7\_avHA and H7N7\_5eqHA to avian  $\alpha$ 2,3-NeuAc sialic acid  
587 receptor types was determined in a solid-phase binding assay using fetuin as a  
588 substrate as previously described (60, 61). The majority of sialic acids in fetuin are  
589 NeuAc and the low amount of NeuGc is neglectable for this assay (62). Briefly, 96  
590 well-plates were coated with 200 $\mu$ l of 10 $\mu$ g/ml fetuin from fetal bovine serum (Merck,  
591 F3004) in 2x PBS overnight at 4°C. Fetuin-coated plates were washed with distilled  
592 water, dried at RT, and coated with 5 log<sup>2</sup> HA units of H7N7\_avHA, H7N7\_5eqHA,  
593 A/quail/California/D113023808/2012 (H4N2) (positive control), or A/Victoria/1975  
594 (H3N2) (negative control) in TBS at 4°C overnight. Viruses were tested in duplicates.  
595 Afterwards, plates were washed with PBS and then blocked for 1h RT using 0.1% de-  
596 sialylated BSA in 2x PBS. BSA was de-sialylated by incubating 5% BSA in 2x PBS +  
597 0,02% penicillin-streptavidin with 1 unit of *Vibrio cholerae* neuraminidase for 24h at  
598 60°C. The plate was washed with washing solution containing 2x PBS + 0.01%  
599 TWEEN80. Horseradish peroxidase (HRP) labeled fetuin was diluted 1:2 in 2x PBS  
600 with 0.02% TWEEN80 + 0.1% de-sialylated BSA and dilutions from 1:200 to 1:12,800  
601 were added to the plate after washing and incubated for 1h at 4°C. After an additional  
602 washing step using washing solution, 100 $\mu$ l peroxidase substrate (Rockland; Lot#  
603 24241) was added at RT. After 30 min the reaction was stopped using 50mM H<sub>2</sub>SO<sub>4</sub>  
604 and the optical density was measured at 450 nm with an ELISA reader (Tecan).

605

## 606 **Plaque test and cell-to-cell spread**

607 Plaque tests were performed using MDCKII for virus titration. Confluent MDCKII cells  
608 were incubated with diluted or undiluted samples for 1h at 37°C with 5% CO<sub>2</sub>. After  
609 infection, cells were washed twice using sterile PBS and overlaid with 1.8% bacto-  
610 agar in Dulbecco's modified Eagle's medium (DMEM) containing 5% bovine serum  
611 albumin (BSA). After incubation for 72 hours, plaque assays were fixed using a 10%  
612 formaldehyde solution with 0.1% crystal violet for 24h. After removal of the agar, the  
613 viral titers were calculated as PFU/ml or PFU/g, or the size of the plaques was  
614 measured under the microscope using Nikon NIS-Elements software.

615

## 616 **Replication kinetics**

617 Different cell cultures were infected with H7N7\_avHA and H7N7\_5eqHA with a  
618 multiplicity of infection (MOI) of 0.001 for 1h. After washing with PBS, the cells were  
619 incubated at 37°C with 5% CO<sub>2</sub>. Cells and supernatants were harvested at indicated  
620 time points and stored at -80°C until the PFU/ml were assessed using plaque tests.  
621 The viral replication in SPF-ECE was tested in 11d-old eggs. Eggs were inoculated  
622 with 100 PFU/0.1 ml of each virus and incubated at 37°C with 56% humidity. Allantoic  
623 fluids were harvested at 8, 24, and 48 hpi and the PFU/ml was determined using  
624 plaque tests.

625

## 626 **Fusion assay to measure pH-dependent HA activation**

627 The HA segments of H7N7\_avHA and H7N7\_5eqHA were cloned into the protein  
628 expression vector pCAGGS by restriction enzyme cloning (H7N7\_avHA\_pcAGGs and  
629 H7N7\_5eqHA\_pcAGGs respectively). A fusion assay was performed to assess the  
630 influence of the equine mutations on the pH activation of the HA as previously  
631 described (35). Briefly, confluent QM-9 cells were transfected in a 24-well plate with  
632 500 ng of H7N7\_avHA\_pcAGGs and H7N7\_5eqHA\_pcAGGs and 100 ng  
633 GFP\_pcAGGs per well using 1µl/µg DNA Lipofectamine 2000 (Thermofisher). PBS  
634 buffers were prepared with pH values ranging from 4.0 to 6.0 (0.2 steps). Transfected  
635 cells were incubated for 16h at 37°C with 5% CO<sub>2</sub> and each well was washed with a  
636 different pH-adjusted PBS buffer for 10 min at RT after incubation. Cells were  
637 incubated for another 4h at 37°C with 5% CO<sub>2</sub> and then fixed with 4%

638 paraformaldehyde for 30 min at RT. The sizes of the fusion events were measured  
639 using a microscope and Nikon NIS-Elements software.

640

### 641 **Thermostability**

642 The thermostability of H7N7\_avHA and H7N7\_5eqHA viruses was tested in a  
643 thermostability assay at 56°C. Allantoic fluid aliquots containing 10<sup>5</sup>PFU/ml of viruses  
644 were prepared in tubes and incubated at 56°C. Samples were taken at 0, 1, 2, 4, and  
645 6 hours post incubation. The infectivity of heat-exposed viruses was assessed in a  
646 plaque test on MDCKII cells. The PFU/ml are shown as mean values from two  
647 independent experimental rounds.

648

### 649 **Animal experiments**

650 For the assessment of the pathogenicity index, nine chickens were infected  
651 intravenously (IV) and ten Pekin ducks were infected intramuscular (IM) with both  
652 recombinant viruses. Daily clinical scoring and the calculation of the pathogenicity  
653 index were performed according to the standard protocol of the world organization for  
654 animal health (WOAH). In addition, ten Pekin ducks and ten chickens were inoculated  
655 via the oculonasal route with 10<sup>5</sup> PFU/ml. One day post-infection (dpi) five contact  
656 birds were added to each group to assess bird-to-bird transmission. In addition to the  
657 daily clinical scoring, oral and cloacal swab samples were taken on 2, 4, 7, and 10 dpi.  
658 The oral and cloacal swab samples were stored in MEM (H) + MEM (E) + NEA medium  
659 with BSA containing enrofloxacin, lincomycin, and gentamicin at -70°C until further  
660 use. The swab samples were titrated in plaque tests on MDCKII cells to assess the  
661 PFU/ml in the collected swabs. Organ samples were collected 4 dpi from three freshly  
662 dead or euthanized birds to assess viral distribution. Organ samples from the brain,  
663 heart, lung, liver, jejunum, spleen, and kidney were lysed in PBS using a tissue lyser  
664 and the PFU/g was assessed using plaque tests on MDCKII cells. The surviving birds  
665 were culled on day ten of the trial and blood was collected for serum samples. The  
666 serum was tested for influenza A NP antibody using a competitive ELISA kit (ID Screen  
667 Influenza A Antibody Competition Multispecies; IDvet).

668



## 669 **Expression and purification of HA for binding studies**

670 HA encoding cDNAs of A/turkey/Italy/214845/02 H7N3 (63) (synthesized and codon-  
671 optimized by GenScript), A/duck/Australia/341/1983 H15N8 (a kind gift from Keita  
672 Matsuno), A/Vietnam/1203/2004 H5N1 (synthesized and codon-optimized by  
673 GenScript), A/chicken/Ibaraki/1/2005 H5N2 (43), and A/Taiwan/2/2013 H6N1 were  
674 cloned into the pCD5 expression vector as described previously (64). The pCD5  
675 expression vector was adapted to clone the HA-encoding cDNAs in frame with DNA  
676 sequences coding for a secretion signal sequence, the Twin-Strep  
677 (WSHPQFEKGGGSGGGSSWHPQFEK); IBA, Germany), a GCN4 trimerization  
678 domain (RMKQIEDKIEEIESKQKKIENEIARIKK), and a superfolder GFP (65) or  
679 mOrange2 (66). Mutations in HAs were generated by site-directed mutagenesis  
680 (primers are available upon request). The HAs were purified from cell culture  
681 supernatants after expression in HEK293S GnTI(-) cells as described previously (64).  
682 In short, transfection was performed using the pCD5 expression vectors and  
683 polyethyleneimine I. The transfection mixtures were replaced at 6 h post-transfection  
684 by 293 SFM II expression medium (Gibco), supplemented with sodium bicarbonate  
685 (3.7 g/L), Primatone RL-UF (3.0 g/L, Kerry, NY, USA), glucose (2.0 g/L), glutaMAX  
686 (1%, Gibco), valproic acid (0.4 g/L) and DMSO (1.5%). At 5 to 6 days after transfection,  
687 tissue culture supernatants were collected and Strep-Tactin sepharose beads (IBA,  
688 Germany) were used to purify the HA proteins according to the manufacturer's  
689 instructions.

690

## 691 **Glycan microarray binding of HA proteins**

692 A selection of a glycan array that was presented elsewhere (67) was used and the full  
693 list of glycans is presented in Fig. S1. When fucosidase treatment was performed,  
694 cells were treated overnight at 37°C with 200 µg/ml fucosidase in fucosidase buffer  
695 (50 mM citrate buffer + 5 mM CaCl<sub>2</sub>, pH 6.0). Anti-sialyl-LewisX antibodies (mouse  
696 IgM, #551344, clone CSLEX1, BD Biosciences) at 50 µg/mL in 40 µL PBS-T were  
697 applied to the subarrays for 90 min. Subsequently, the arrays were incubated with a  
698 mixture of goat anti-mouse IgM HRP (10 µg/mL; #1021-05 Southern Biotech) and  
699 donkey anti-goat antibody labeled with AlexaFluor555 (5 µg/mL; A21432, Invitrogen)  
700 in 40 µL PBS-T for 1 h. HAs were pre-complexed with human anti-streptag and goat  
701 anti-human-AlexaFluor555 antibodies in a 4:2:1 molar ratio, respectively in 50 µL PBS-

702 T on ice for 15 min. The mixtures were added to the subarrays for 90 min in a  
703 humidified chamber. Wash steps after each incubation (e.g. enzyme treatment, HA,  
704 or antibody incubation) involved six successive washes of the whole slides with either  
705 twice PBS-T, twice PBS, and twice deionized water. The arrays were dried by  
706 centrifugation and immediately scanned as described previously (27). Processing of  
707 the six replicates was performed by removing the highest and lowest replicates and  
708 subsequently calculating the mean value and standard deviation over the four  
709 remaining replicates.

710

### 711 **Fucosidase production**

712 The protein sequence of fucosidase E1\_10125 from *Ruminococcus gnavus* E1 (56)  
713 was obtained from the RCSB Protein Data Bank under accession number 6TR3. The  
714 nucleotide sequence was obtained from the closest hit in a protein-protein search in  
715 BLAST, of which the nucleotide sequence was corrected to obtain the exact same  
716 amino acid sequence. This open reading frame was ordered at GenScript and cloned  
717 into backbone pET23A in frame with a His-tag on the C-terminus of the open reading  
718 frame of the fucosidase. Cloning was performed in JM109 *Escherichia coli* (Promega).  
719 The plasmid is deposited at addgene under the name: pET23A-Fucosidase-  
720 E1\_10125-Ruminococcus\_gnavus-His (#207665). Expression of the fucosidase was  
721 performed in BL21 (DE3) *E. coli* (New England Biolabs). An overnight culture (90 ml  
722 per liter culture) was inoculated in 2xYT medium (Serva) supplemented with 50 µg/ml  
723 ampicillin (13398.02, Serva). Bacteria were grown at 37°C while shaking at 200 rpm  
724 until OD 0.8-1.0, after which fucosidase production was induced with 1mM isopropyl  
725 β-d-1-thiogalactopyranoside (R0309, Invitrogen). Afterward, the bacteria were grown  
726 for 21 h at room temperature while shaking at 200 rpm. Cell pellets were obtained by  
727 centrifugation in a swing-out centrifuge for 30 min at 4°C at 629 rcf. The pellets were  
728 resuspended in 50ml lysis buffer (100mM Tris-HCl, pH 8.0, 0.1% TritonX-100) per liter  
729 bacterial culture, which was supplemented with 1 gram per liter bacterial culture  
730 lysozyme (62971, Merck), 25 µl per liter bacterial culture DNase (EN0521, Thermo  
731 Fisher Scientific), and 25 µl per liter bacterial culture DNase buffer. The mixtures were  
732 incubated for 50 minutes at 37°C while shaking at 200 rpm. The cells were additionally  
733 lysed by sonication (Bandelin, Sonopuls, needle MS73) at 50% amplitude, three times  
734 for one minute at 10s intervals. The lysates were centrifuged for 1.5 h at 4°C at 629

735 rcf until the supernatant was clear. The supernatant was filtered through a 0.45  $\mu$ m  
736 filter (431220, Corning) and incubated for 16h with Ni-NTA beads at 4°C while rotating.  
737 The beads were washed using a washing buffer (0.5M NaCl, 20mM Tris-HCl, pH 7.5)  
738 after which the fucosidase was eluted using the same buffer supplemented with 10-  
739 200mM imidazol. The elutions were concentrated and the buffer was exchanged to  
740 fucosidase buffer (50 mM citrate buffer + 5 mM CaCl<sub>2</sub>, pH 6.0) using a centrifugal  
741 concentrator with a molecular weight cutoff of 10 kDa (Vivaspin 6, VS0602, Sartorius).  
742 The presence of the fucosidase (62 kDa) was evaluated by running an SDS-PAGE gel  
743 (after denaturing for 15 min at 95°C with the addition of denaturing buffer (NP0009,  
744 Invitrogen)) with consequent staining using Coomassie blue dye.

745

### 746 **Protein histochemistry**

747 Sections of formalin-fixed, paraffin-embedded chicken (*Gallus gallus domesticus*)  
748 were obtained from the Division of Pathology, Department of Biomolecular Health  
749 Sciences, Faculty of Veterinary Medicine of Utrecht University, the Netherlands.  
750 Sections of Pekin ducks were obtained from the animal experiment that is described  
751 above. Protein histochemistry was performed as previously described (68, 69). In  
752 short, tissue sections of 4  $\mu$ m were deparaffinized and rehydrated, after which  
753 antigens were retrieved by heating the slides in 10 mM sodium citrate (pH 6.0) for 10  
754 min. Endogenous peroxidase was inactivated using 1% hydrogen peroxide in MeOH  
755 for 30 min at RT. Slides were treated overnight at 37°C with 150  $\mu$ g/ml fucosidase in  
756 the fucosidase buffer (50 mM citrate buffer + 5 mM CaCl<sub>2</sub>, pH 6.0) or buffer only.  
757 Subsequently, slides were washed with PBS with 0.1% Tween-20 (PBS-T). Tissues  
758 were blocked for 90 min at RT using 3% BSA (w/v) in PBS. Anti-sialyl-LewisX  
759 antibodies (mouse IgM, #551344, clone CSLEX1, BD Biosciences) were diluted  
760 1:1000 in PBS and precomplexed with goat anti-mouse IgM-HRP (#1021-05, Southern  
761 Biotech) in a 1:100 dilution on ice for 20 min. The slides were stained for 90 min with  
762 pre-complexed HAs as previously described for the glycan microarray or the anti-  
763 sialyl-LewisX mixtures. For WT H5 HA, we used 1.5  $\mu$ g/ml HA, for H5 R222K R227S  
764 HA, we used 3  $\mu$ g/ml HA, for H7 HAs, we used 2  $\mu$ g/ml HA, and for H15 HA, we used  
765 1  $\mu$ g/ml HA. After washing with PBS, binding was visualized using 3-amino-9-  
766 ethylcarbazole (AEC) (Sigma-Aldrich, Germany) and slides were counterstained using  
767 hematoxylin.

768

## 769 **Identification of *N*-glycans on cells by mass spectrometry**

770 Cell lysates were obtained as described above. Preparation of mass spectrometry  
771 samples and measuring of these samples was performed as described previously  
772 (59). Briefly, glycans from cell lysates were released using PNGaseF treatment,  
773 labeled with procainamide 2-picoline borane, and purified in multiple steps. The  
774 samples were measured both using positive mode HILIC-IMS-QTOF analysis and  
775 MS/MS (using a Thermo Scientific Exploris 480 connected to a Thermo Scientific  
776 Ultimate 3000 UPLC system).

777

778 IM-MS data was calibrated to reference signals of  $m/z$  121.050873 and 922.009798  
779 using the IM-MS reprocessor utility of the Agilent Masshunter software. The mass-  
780 calibrated data was then demultiplexed using the PNNL preprocessor software using  
781 a 5-point moving average smoothing and interpolation of 3 drift bins. To find potential  
782 glycan hits in the processed data, the 'find features' (IMFE) option of the Agilent IM-  
783 MS browser was used with the following criteria: 'Glycans' isotope model, limited  
784 charge state to 5 and an ion intensity above 500. The found features were filtered by  
785  $m/z$  range of 300 – 3200 and an abundance of over 500 (a.u.) where abundance for a  
786 feature was defined as 'max ion volume' (the peak area of the most abundant ion for  
787 that feature). After exporting the list of filtered features, glycans with a mass below  
788 1129 Da (the mass of an *N*-glycan core) were removed. The ExPASy GlycoMod tool  
789 (70) was used to search for glycan structures (monoisotopic mass values, 5 ppm mass  
790 tolerance, neutral, derivatized N-linked oligosaccharides, procainamide (mass  
791 235.168462) as reducing terminal derivative, looking for underivatized  
792 monosaccharide residues (Hexose, HexNAc, Deoxyhexose, and NeuAc)). For  
793 features with multiple potential monosaccharide combinations, the most realistic  
794 glycan in the biological context was chosen. The abundance of glycan features with  
795 the same mass, composition, and a maximum difference of 0.1 min in the retention  
796 time were combined as one isomer. A full glycan composition feature list for MDCK-  
797 FUT cells is presented in Table S2. Analysis of the number of fucoses per sialylated  
798 glycan was performed on the complex and hybrid *N*-glycans with at least one sialic  
799 acid.

800

801 For MS/MS data, Proteowizard MSconvert (version 3.0.21328-404bcf1) was used to  
802 convert Thermo raw files to MGF format using MGF as output format, 64-bit binary  
803 encoding precision and with the following options selected: write index, zlib  
804 compression and TPP compatibility. No filters were used when converting raw files to  
805 MGF format. To search MGF files for spectra containing glycan oxonium ions an  
806 internally developed tool named Peaksuite (v1.10.1) was used with an ion delta of 20  
807 ppm, noise filter of 0% and using a list of oxonium  $m/z$  values as mass targets (Table  
808 SX from (59)). Scans without any detected peaks were removed. Python 3.2.2 was  
809 used for data curation based on precursor  $m/z$  (10 ppm), retention time (17-24 min)  
810 and intensities of oxonium ions that originated from the glycan core ( $m/z$  441.2707,  
811 587.3286, 644,3501, 790.4080, 806.4029, and 952.4608). The sum intensity threshold  
812 of the core oxonium ions was set to  $1e4$ . Python 3.2.2 was also used for calculating  
813 the relative intensities of oxonium ions corresponding to sLe<sup>x</sup> ( $m/z$  803.2928)  
814 normalized versus the sum intensities of the core oxonium ions.

815

### 816 **Flow cytometry studies**

817 Detaching of the cells (MDCK WT and MDCK-FUT) was performed with 1X TrypLE  
818 Express Enzyme (12605010, Thermo Fisher Scientific), using 2 ml in a T75 flask, at a  
819 confluency of approximately 90%. Cell pellets were obtained by centrifugation for 5  
820 min at 250 rcf, after which the cells were resuspended in PBS supplemented with 1%  
821 FCS (S7524, Sigma) and 2mM EDTA and kept at 4°C until the plate was measured in  
822 the flow cytometer. In a round-bottom 96-well plate (353910, Falcon), 50,000 cells per  
823 well were used. Per well, 1 µg of precomplexed HA or precomplexed anti-sialyl-LewisX  
824 antibody (CD15S, clone CSLEX1, #551344, BD Biosciences) was added to PBS  
825 supplemented with 1% FCS and 2mM EDTA to achieve a final concentration of 20  
826 µg/ml. Precomplexation of HAs was performed with 1.3 µg monoclonal antibody  
827 detecting the Twin-Strep-tag and 0.325 µg goat anti-human Alexa Fluor 488 (A11013,  
828 Invitrogen) and incubated on ice for 20 min. Precomplexation of the anti-sialyl-LewisX  
829 antibody was performed with a 1:50 dilution of goat anti-mouse IgM-HRP (#1021-05,  
830 Southern Biotech) and 0.65 µg donkey anti-goat Alexa Fluor 555 (A21432, Invitrogen)  
831 and incubated on ice for 20 min. Furthermore, eBioscience Fixable Viability Dye eFluor  
832 780 (65-0865, Thermo Fisher Scientific) was diluted 1:2000 in the same mixture. Cells  
833 were incubated with the hemagglutinin/antibody mix for 30 minutes at 4°C in the dark.

834 Cells were washed once with PBS supplemented with 1% FCS and 2 mM EDTA, after  
835 which the cells were fixed with 100  $\mu$ l of 1% paraformaldehyde in PBS for 10 minutes.  
836 Afterward, cells were washed twice using PBS supplemented with 1% FCS and 2 mM  
837 EDTA, after which they were resuspended in 100  $\mu$ l of the same buffer. Flow cytometry  
838 was performed using the BD FACSCanto II (BD Biosciences) using appropriate laser  
839 voltages. Alexa Fluor 488 signal (HAs) was measured using the FITC filter and Alexa  
840 Fluor 555 signal (anti-sialyl-LewisX signal) was measured using the PE filter. Data  
841 were analyzed using FlowLogic (Inivai Technologies) and gated for cells, single cells,  
842 and alive cells as described previously (59). The mean value and standard deviation  
843 were calculated over triplicate measurements.

844

### 845 **Sequence analysis**

846 The prevalence of the five equine amino acid residues on positions 128, 130, 135,  
847 189, and 193 (numbering according to H3 HA) was analyzed in all equine and avian  
848 H7 sequences with a minimum length of 1,600 bp. The protein sequences were  
849 downloaded from GISAID (date of download: 31.08.2023) and aligned using MAFFT  
850 package in Geneious software. Only the receptor binding site of the different  
851 sequences was compared. In total 3,402 avian H7 and 24 equine H7 sequences were  
852 analyzed.

853

### 854 **Data analysis and statistical analysis**

855 The data in this article were (statistically) analyzed and visualized using GraphPad  
856 Prism 9.2.0.

857

### 858 **Data deposition**

859 The released *N*-glycan raw data (for the glycomic analyses of the cell lines) have been  
860 deposited to the GlycoPOST repository (Watanabe, Y., Aoki-Kinoshita, K.F., et al.  
861 2021) under the identifier GPST000345.

862

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871 that were used for analysis in this study.

872

### 873 **Ethics statement**

874 All performed animal experiments were approved and monitored by the commissioner  
875 for animal welfare at the Friedrich-Loeffler-Institut (FLI), representing the Institutional  
876 Animal Care and Use Committee (IACUC). The animal trial was performed under  
877 biosafety level 3 (BSL3) conditions in the animal facilities of FLI according to the  
878 German Regulations for Animal Welfare. The experiments were approved by the  
879 authorized ethics committee of the State Office of Agriculture, Food Safety, and  
880 Fishery in Mecklenburg-Western Pomerania (LALLF M-V) under registration number  
881 7221.3–1.1-051-12. The specific-pathogen-free (SPF) embryonated chicken eggs  
882 (ECE) used for this publication were purchased from Valo BioMedia (Germany). The  
883 storage and handling of SPF-ECE were performed according to the guidelines of the  
884 World Organization for Animal Health (WOAH).

885

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894 de Vries).

895 **Supplementary information**

896 **Table S1. Prevalence of different amino acid residues in avian and equine H7 HA sequences.**

897 Sequences with a size of at least 1,600bp were downloaded from GISAID on 31.08.2023 and analyzed  
898 using Geneious software.

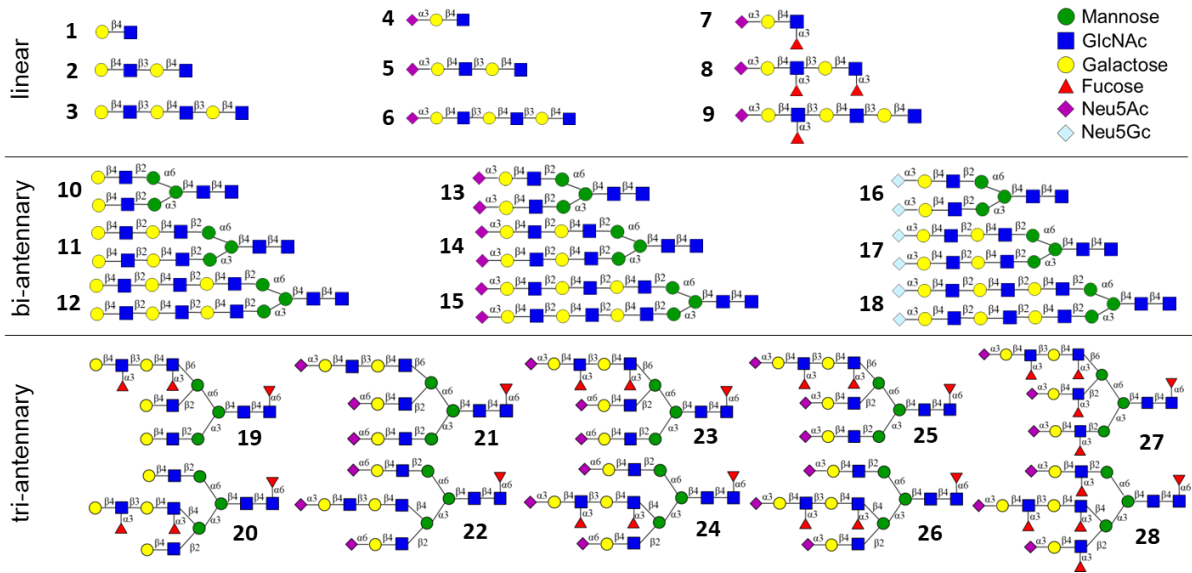
HA position	Amino acid	Avian H7 (n=3,402)		Equine H7 (n=24)	
		No.	%	No.	%
128	S	2,887	84.86	-	-
	T	2	0.06	24	100
	N	487	14.32	-	-
	D	20	0.59	-	-
	G	3	0.09	-	-
	I	1	0.03	-	-
	Y	1	0.03	-	-
	R	1	0.03	-	-
130	I	3,356	98.65	-	-
	V	8	0.24	24	100
	M	38	1.12	-	-
135	A	2,574	75.66	-	-
	E	8	0.24	24	100
	V	522	15.34	-	-
	T	295	8.67	-	-
	G	1	0.03	-	-
	K	1	0.03	-	-
	S	1	0.03	-	-
189	T	1,752	51.50	-	-
	A	1,297	38.12	24	100
	S	168	4.94	-	-
	D	93	2.73	-	-
	E	43	1.26	-	-
	N	41	1.21	-	-
	I	5	0.15	-	-
	G	1	0.03	-	-
	K	1	0.03	-	-
	Q	1	0.03	-	-
193	K	3,360	98.77	1	4.17
	R	37	1.09	23	95.83
	M	3	0.09	-	-
	N	1	0.03	-	-
	Q	1	0.03	-	-

899



900 **Table S2. Relative abundance of N-glycans of MDCK FUT cells measured using HILIC-IMS-QTOF**  
 901 **positive mode mass spectrometry.** The table is presented in an additional excel file. The data from  
 902 MDCK WT cells is published in Table SII of (59).

903  
 904 **Figure S1. Glycans presented on the glycan microarray**



905

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