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Viral Determinants of Reliance on Multiple Infection in Influenza A Viruses

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B.S., The University of Virginia's College at Wise, 2013

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Abstract

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Viral infections involving two or more virions in a single cell have been seen to impact infection outcomes for a diverse array of virus types. We previously found that influenza A/guinea fowl/Hong Kong/WF10/99 (H9N2) virus (GFHK99) displays a particularly high reliance on multiple infection in mammalian cells tied to the PA gene segment. This dissertation sought to uncover the viral processes underlying the high reliance phenotype of GFHK99. Herein, the experimental techniques and data collected in the evaluation of GFHK99 are described. PA 26K was found to suppress endonuclease activity and viral transcription, specifically within cells infected at low multiplicity. The model arising from this research postulates that sub-optimal activity of the GFHK99 endonuclease results in inefficient priming of viral transcription, an insufficiency which can be overcome with the introduction of additional viral templates to the cell. These findings add to the burgeoning field of collective cellular interactions of viruses and have implications for continued public health surveillance of H9N2 IAVs.

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To my fiancée, Jakob: We met in immunology class but the chemistry was undeniable. I have been and always shall be yours.

This work is dedicated to my mother, B’Lynn.

She gave me my love of science and I am heartbroken that she couldn’t see me graduate.

I know she would have been so proud.

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Chapter 1

Introduction:

Influenza is a respiratory illness occurring commonly in humans in outbreaks that can be traced back centuries, perhaps even to ancient Greece. Long before the causative viruses were isolated, widespread infections "*influenza di freddo*," "caused by cold," repeatedly spread across the globe (9, 10). In 1918 and 1919, tens of millions of people were killed during the largest known influenza pandemic, but it wasn't until the 1930s that scientists isolated a virus from nasal secretions that was found responsible (11). Since then, numerous types and strains of influenza have been identified and extensive research has been done to characterize them, but influenza still has an enormous impact on human health and agriculture each year that necessitates further work to understand the dynamics of infection.

Virus structure and lifecycle

IAV structure

Influenza A virus (IAV) is a member of the *Orthomyxoviridae* family of enveloped RNA viruses that also includes influenza B, C, and D viruses (12). While influenza C virus and influenza D virus are not major concerns for humans, IAV and influenza B (IBV) viruses circulate in humans and cause illness in seasonal outbreaks. IAV, in particular, has the largest impact on public health, the broadest genetic diversity, and can infect a wide range of animals. The negative-sense, approximately 13 kilobase genome of IAV is divided over eight segments that encode at least eleven viral proteins (13). RNA segments are packaged into viral ribonucleoproteins (vRNPs) composed of a strand of negative sense RNA associated via its phosphate backbone with multiple units of viral nucleoprotein (NP) and one unit of a heterotrimeric viral polymerase (Fig. 1). The

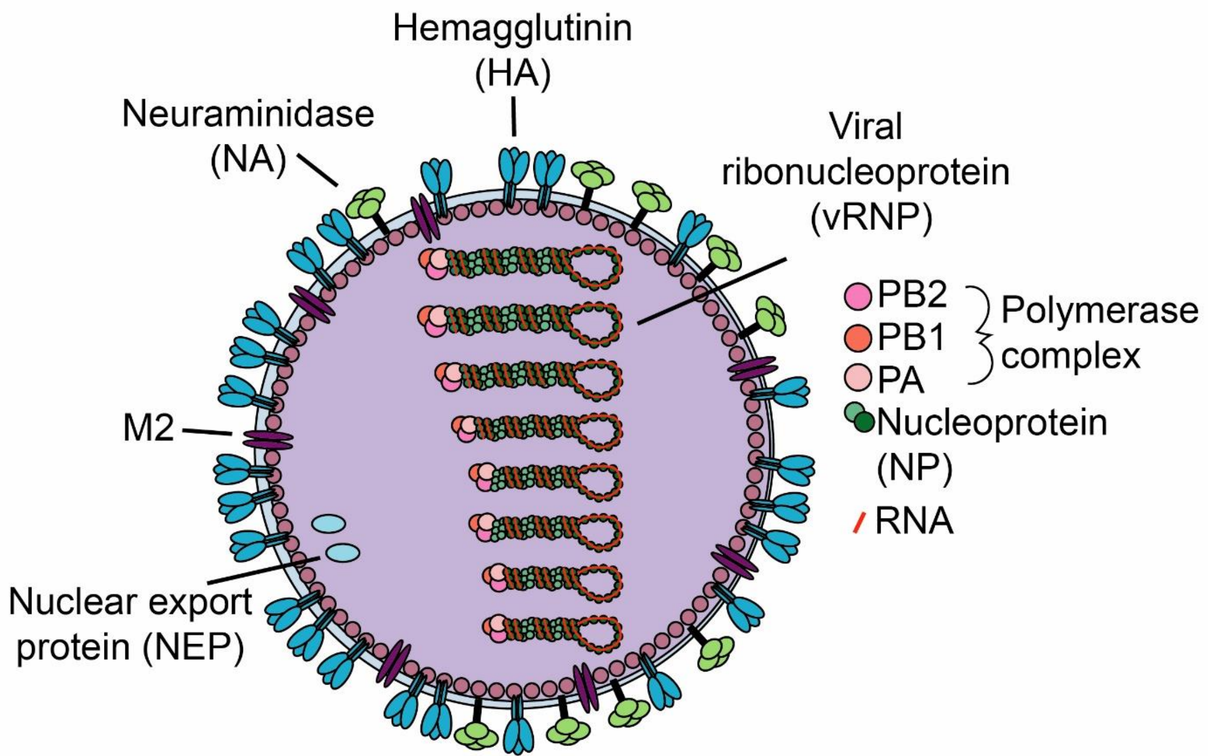

Fig. 1: The structure and proteins of an influenza A virion. This simplified cartoon shows the general location and format of each of the viral proteins encoded by IAVs (2, 6). (Graphic: J. Shartouny)

24 ends of each segment are complementary and anneal, forming a loop that then twists into an anti-
25 parallel helix by associating NPs (10). Generally, eight vRNPs are incorporated per virion and are
26 arranged in a 7+1 pattern where one vRNP is in the center of the array with the seven other vRNPs
27 surrounding it in a circle (11). Matrix proteins (M1) form a shell around vRNPs and copies of the
28 nuclear export protein (NEP). Surrounding the matrix is a lipid bilayer derived from host cell
29 membranes that is studded with transmembrane viral proteins. The most abundant protein in the
30 envelope is the homotrimeric glycoprotein hemagglutinin (HA) which facilitates host cell entry
31 and fusion. The mushroom-shaped homotetramer neuraminidase (NA) and a proton channel (M2)
32 are the other two envelope proteins (9, 12).

33 IAVs are classified into subtypes named for the combination of HA and NA, the surface
34 antigens that each possesses. There are 18 different HA types and 11 NA types currently defined
35 that can exist in varied combinations. Each identified HA and NA type has been found in birds
36 except H17, H18, N10, N11 which have exclusively been identified in bats (14, 15). In humans,
37 viruses of the IAV subtypes H1N1, H2N2, and H3N2 have circulated widely, and H1N1 and H3N2
38 are the current circulating strains that cause seasonal illness each year. Other subtypes of IAV that
39 circulate in birds, including H5N1 and H7N9, have been known to sporadically infect humans.
40 IAV subtypes are divided further into genetically-related groups, or clades, which have a single
41 common ancestor.

42 A systematic naming convention is employed to facilitate identification of influenza
43 viruses. Strain names indicate the influenza type (A, B, C, D), the location where the virus was
44 sampled, the strain number, the year collected, and the HA and NA subtype in parentheses. If the
45 virus is isolated from a non-human host, the species is also noted. For example, influenza A/Puerto
46 Rico/8/1934 (H1N1) virus, a commonly used lab strain, is a human-derived IAV isolated in Puerto
47 Rico in 1934 whereas another common lab strain, influenza A/duck/Hunan/X38/2012 (H3N2)
48 virus, is a duck-derived virus from Hunan, China.

49

50 *Influenza A virus lifecycle*

51 An infection with IAV begins with the attachment of the HA on the virus to terminal sialic
52 acid moieties on glycans on the surface of host cells. Receptor-mediated endocytosis brings the
53 virion into the cell in an endosome and, as the endosome decreases in pH, entry into the cell is
54 facilitated by HA (16). Low pH triggers a conformational change in HA that leads to exposure of
55 a buried viral fusion peptide and subsequent insertion into the endosomal membrane. Additional

56 conformational changes in HA draw the viral envelope and endosomal membrane together leading
57 to fusion. Protons are also pumped into the interior of the virion through the M2 channel, which
58 leads to the dissociation of the M1 matrix. vRNPs are then released into the cytoplasm of the cell
59 (17). Nuclear localization signals on the NP proteins in the vRNP complexes are recognized by
60 host cell importin proteins which transport them through nuclear pores into the cell's nucleus (12).

61 Transcription of influenza genes occurs in the nucleus. The polymerase trimer in the
62 vRNPs, which acts as an RNA-dependent RNA polymerase (RdRp), synthesizes viral mRNA.
63 Host cell 5' m^7G mRNA caps are pilfered from nascent cellular transcripts to prime viral mRNA.
64 The viral polymerase associates with the C-terminal domain of the cellular protein RNA
65 polymerase II, which caps host mRNA transcripts. PB2 binds the caps while PA endonuclease
66 cleaves 10-15 nucleotides downstream (16, 18). PB1 then extends the mRNA transcript in its
67 central chamber using the vRNA as a template, stuttering on a stretch of uridines near the end of
68 the vRNA to produce a poly(A) tail(17).

69 Capped and tailed viral mRNAs are exported from the nucleus by cellular mechanisms and
70 translated by host cell ribosomes. The transcripts of the influenza surface proteins, HA, NA, and
71 M2, are processed by endoplasmic reticulum-associated ribosomes, where they are inserted into
72 the membrane, folded, and oligomerized. These are then trafficked to the plasma membrane of the
73 cell. The remaining influenza transcripts are translated by cytosolic ribosomes. Newly-synthesized
74 proteins are chaperoned back into the nucleus by cellular importin where they then participate in
75 further transcription or replication (19).

76 Replication of viral RNA segments consists of two steps mediated by the influenza
77 polymerase complex. First, vRNA is copied into complementary RNA (cRNA), then the cRNA is
78 used to template new vRNA. cRNA synthesis does not require priming in the same manner as viral

79 mRNA synthesis. Instead, the polymerase bound to the vRNA initiates replication at the terminus
80 of the 3' untranslated region by synthesizing a phosphorylated AG dinucleotide that is stabilized
81 by a priming loop within PB1 (20-22). As cRNA is synthesized, it is bound by a second polymerase
82 complex and NP to form a cRNP (19). vRNA synthesis proceeds from the cRNP using internal
83 initiation (23). vRNAs are bound by a third polymerase complex and NP is recruited to form
84 vRNPs.

85 Each component of a virus must be transported to the host plasma membrane for assembly
86 and release from the cell. HA, NA, and M2 are transported to the apical cell membrane and
87 accumulate near areas dense in cholesterol and sphingolipids, called lipid rafts. Within the nucleus,
88 vRNPs associate with M1, which binds NEP, which coordinates exit from the nucleus through
89 interactions with host proteins CRM-1 and Ran-GTP. These host proteins move vRNP+M1+NEP
90 through pores in the nuclear membrane and release the complex into the cytoplasm. Subsequent
91 trafficking toward the plasma membrane occurs via recycling endosomes, which are used by the
92 cell to return membrane components that have been internalized back to the cell surface (24). The
93 host protein Rab11, a small GTPase, regulates recycling within a cell and moves cargo to the
94 plasma membrane (25). vRNPs are recruited to Rab11+ endosomes and are transported via
95 microtubules to the plasma membrane where M1 can associate with the intracellular M2 and
96 concentrate the influenza components at the surface (11).

97 Budding of influenza viruses from an infected cell's surface requires bending and scission
98 of the plasma membrane studded with influenza proteins. While HA and NA expression alone can
99 lead to budding, M1 plays a role in the morphology of particles and M2 aids in curvature of the
100 membrane (26). Release of budded viruses from the cell surface depends on NA, which catalyzes

101 the removal of local sialic acid from the cell and virus surfaces to prevent the HA on budding
102 particles from sticking to the originating cell or from aggregating (27, 28).
103
104 *Genetic diversity*
105 A feature of influenza virus that contributes to its persistent circulation is antigenic
106 variation (29). Influenza viruses can differ in their genetic makeup, protein sequences, and surface
107 structures. Despite infection eliciting a strong immune response, a person can be infected with
108 influenza multiple times
109 as they encounter viruses
110 with different HA and
111 NA surface proteins
112 toward which their
113 previously-established
114 anti-influenza immune
115 response is ineffective.
116 The starting point that
117 leads to diversity of
118 influenza viruses can be
119 attributed to two
120 mechanisms of genetic
121 change: mutation and
122 reassortment (Fig. 2).

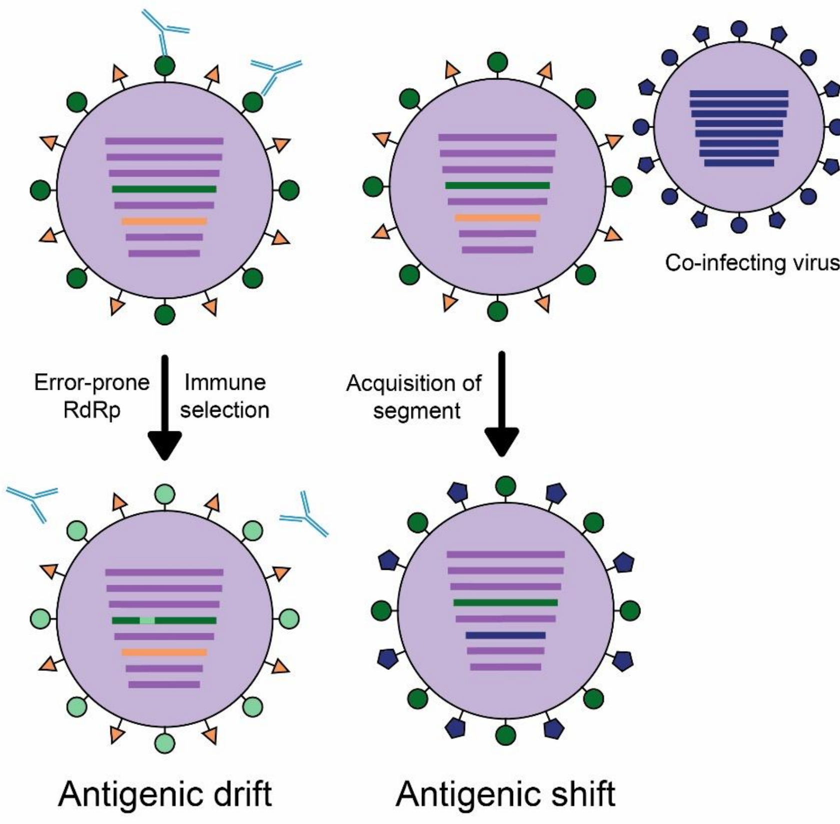

Fig. 2: Antigenic drift and antigenic shift. Influenza viruses have two main mechanisms of genetic change, mutation and reassortment. Drifting of antigenic sites occurs when mutations in the genome become fixed by antibody-mediated selection. A major shift in antigenic type can occur when segments from two heterologous IAVs mix during reassortment (2, 7, 8). (Graphic: J. Shartouny)

123 Nucleotide mutations during the replication of the viral genome can become fixed in a viral
124 population by antibody mediated selection. The gradual change of influenza virus genetic code is
125 referred to as antigenic drift. The virally-encoded RdRp does not have the ability to proofread and
126 edit the nucleotides that it adds as it synthesizes new chains RNA so new genomes lack fidelity to
127 the originally-infecting genome (8). An estimated 2 to 3 mutations are incorporated per replicated
128 genome (30). Most mutant viruses are either unfit for transmission or, through intra- and inter-host
129 bottlenecks, never get the chance to transmit (31). Mutations resulting in viable viruses, however,
130 tend to cluster in the antigenic sites of surface proteins, which, over time, can lead to the emergence
131 of viruses that can escape humoral immune responses in infected hosts since the antibodies cannot
132 bind to the surface proteins as effectively as they could the virus toward which their initial response
133 was garnered (32). This leads to the selection of these drifted viruses and replacement of the
134 previously-circulating viruses with drifted viruses circulating through a population within several
135 seasons (8, 31, 33).

136 Reassortment is a sudden change in the IAV genome where one or more of the gene
137 segments of a virus is incorporated into the genome of another virus that has infected the same
138 cell. With IAV's eight segments, 256 combinations of segments exchanged between two viruses
139 are theoretically possible, however the ultimate diversity generated through reassortment is
140 reduced via negative selection in subsequent rounds of replication (32, 34). Reassortment of NA
141 and HA segments can lead to a dramatic change in antigenicity, called antigenic shift, which can
142 pose a significant threat for interspecies transmission. A pig, for example, coinfected with a swine
143 virus as well as an avian virus could produce a reassortant that is adapted to mammalian
144 transmission with avian-derived surface antigens (35). Reassortment events like this can cause

145 pandemics where a novel virus spreads quickly through a population with no previous immunity
146 (36).
147
148 ***IAV ecology and impact***
149
150 *IAV in avian hosts*
151 IAV is known to infect a large number of species which, aside from wild birds, poultry,
152 pigs, and humans, includes bats, seals, horses, and dogs among others. Transmission between
153 species is sporadic but sustained intra-species circulation has been established in a number of these
154 hosts (37, 38). The initial and largest reservoir for IAV circulating in most species is wild aquatic
155 birds, especially in the orders of Anseriformes, which includes ducks, and Charadriiformes, which
156 includes gulls (35). Nearly every combination of HA and NA subtypes have been found in birds
157 (36)(39). Susceptible wild birds exist all over the globe and many waterfowl species are migratory,
158 following diverse migratory paths that can bring them to new locales and into contact with new
159 populations of susceptible birds which can then disperse, carrying viruses with them.
160 The pathogenesis of IAV in birds differs from what is commonly seen in human influenza
161 infections. The preferred receptor for avian viruses is on the surface of cells in gastrointestinal tract
162 so these viruses are confined largely to this area and are primarily spread through the fecal-oral
163 route (40). Viruses can remain viable in water for up to a month, depending on the temperature,
164 which creates an effective means of transmission between birds that spend time in and around
165 bodies of water (41). Infection is thought to be largely asymptomatic or mild and new viruses are
166 shed in the feces into the environment. The prevalence of viruses in sampled populations varies by
167 species, season, and geographic location as well as the number of susceptible individuals (42, 43).

168 Domestic birds like chickens and turkeys can also be infected by IAVs via transmission
169 from wild birds through contaminated water sources or contact. These viruses can adapt to the
170 poultry species and become established in the population. Most IAVs affecting poultry can be
171 classified as low-pathogenic avian influenza (LPAI), causing mild symptoms with low
172 mortality(44). Infection can occur in the nasal cavity, respiratory tract, GI tract, and oviduct,
173 limited by the location of essential proteases that are expressed only in certain tissues, generally
174 on surface epithelia in the airway and digestive tract (45, 46).

175 Some subtypes of influenza virus are known as high-pathogenic avian influenza (HPAI)
176 because they cause increased morbidity and mortality in chickens (47). These viruses have a
177 modified cleavage site that can be targeted by proteases that are expressed widely throughout the
178 body, leading to a more systemic infection and higher lethality. Viruses of H5 and H7 subtypes
179 that are LPAI can acquire the modified cleavage site while circulating in poultry, resulting in a
180 HPAI (48, 49). Large losses of domestic birds have occurred in outbreaks on farms due to high
181 mortality and subsequent flock culling (15).

182 A subset of LPAI influenza viruses, H9N2, has become established in poultry across the
183 globe. First discovered in an outbreak in Wisconsin turkeys in 1966, H9N2 viruses are now
184 endemic in poultry in the Americas, Asia, the Middle East, and North Africa and are classified into
185 an American branch and a Eurasian branch (50, 51). The American branch seems to largely infect
186 wild birds with sporadic spillovers into domestic turkeys. The Eurasian branch, however, has
187 become established in poultry with three identified lineages and are named after their prototype
188 viruses: A/quail/Hong Kong/G1/1997 (G1 lineage), A/chicken/Beijing/1/94 (BJ94 lineage), and
189 A/chicken/Hong Kong/Y439/1997 (Y439 lineage). The G1 lineage is further divided into the
190 Western and Eastern sub-lineages due to geographic variation. Sampling of poultry taken to live

191 markets in several Asian countries has shown high prevalence of exposure and raises concerns of
192 a possible poultry-to-poultry and poultry-to-human infection interface (50). Indeed, humans have
193 been infected with H9N2 viruses, largely in China, and generally present with mild illness (52).
194 To date, no known human-to-human transmission has been identified.

195

196 *IAV in swine hosts*

197 Another domestic animal that serves as a large reservoir for influenza A viruses is pigs.
198 After several decades of speculation that "hog flu" was actually related to human influenza, it was
199 shown in 1931 to be caused by a virus and in 1934 to be similar to human influenza (53). The
200 isolated virus was given the designation H1N1 and the classical H1N1 (cH1N1) was the
201 predominant lineage circulating in pigs in the United States for much of the 20th century (54).
202 Outbreaks in herds spread quickly with high fevers and respiratory symptoms like coughing, but
203 generally low mortality. By the 1970s, it became clear that humans could be infected with swine-
204 originating viruses by studying people who became sick after contact with pigs on farms or in
205 laboratory settings (55).

206 Pigs express the receptors preferred by both human and avian viruses (56). If a pig were to
207 become infected with more than one virus originating from different species, it can lead to the
208 reassortment combining avian and mammalian gene segments. This "mixing vessel" hypothesis
209 places pigs in a position to facilitate the emergence of viruses that are better adapted to a
210 mammalian host than direct zoonosis from a bird. In the 1990s, pigs in North America were found
211 to become infected with a "triple-reassortant" virus, an H3N2 subtype virus that contained genes
212 from humans (HA, NA, PB1) and birds (PB2, PA) as well as swine (53). This virus was then
213 subject to reassortment with cH1N1 to produce several other subtypes that spread through pig

214 populations (54). An avian H1N1 was introduced to swine in Europe in the 1970s, “avian-like” 215 swine virus, and quickly replaced cH1N1 circulation among pigs on the continent (56, 57). In 216 2009, a novel swine-origin virus, “swine flu,” became pandemic in humans and was found to 217 derive from the triple-reassortant with NA and M segments contributed by the Eurasian avian-like 218 swine virus (pdmH1N1) (58, 59).

219

220 *IAV in human hosts*

221 Humans are susceptible to several subtypes of IAVs, albeit many fewer antigenic types 222 than infect birds and pigs. An even smaller number of these, H1N1, H2N2, and H3N2 subtypes, 223 have established sustained transmission between humans. Nevertheless, influenza virus infections 224 are a major public health concern, causing recurring epidemics that lead to tens of thousands of 225 deaths and millions of dollars in healthcare costs globally each year (60, 61).

226 Human-adapted influenza viruses primarily cause respiratory illness. Symptoms can 227 include fever, chills, muscle aches, cough, congestion, and shortness of breath. Young children, 228 individuals with chronic diseases like diabetes, and the elderly are considered especially at risk of 229 developing severe influenza. Vaccines are the most effective method of controlling the spread of 230 influenza between humans and are available in inactivated, live-attenuated, and subunit formats 231 (62, 63).

232

233 *Seasonal outbreaks*

234 “Flu season” has become a common moniker for the late fall and winter, as epidemics of 235 influenza infections track across the globe, infecting large proportions of the population during the 236 colder months. An estimated 3-400,000 influenza deaths occur around the world each year during

237 these epidemics (60). Currently, IAVs of H1N1 and H3N2 subtypes as well as IBV circulate
238 endemically, some of the few influenza virus subtypes to establish lasting transmission chains in
239 humans. The particular strains causing each season's outbreaks differ due to antigenic drift and
240 host immune selection, with emergent variants replacing previous strains.

241 Updated vaccines are recommended each year for individuals 6 months of age or older
242 based on the strains predicted to impact the population the most. The United States Centers for
243 Disease Control and Prevention (CDC) estimates that more than 7 million illnesses and 6,300
244 deaths were averted by the use of vaccines during the 2019-2020 season (64). To allay seasonal
245 illness, three types of influenza vaccines are used annually: inactivated, live-attenuated, and
246 recombinant (62). In all three platforms, the vaccine includes components from three or four
247 influenza virus lineages that circulate endemically: one H1N1 subtype IAV, one H3N2 subtype
248 IAV, and one or both of the Yamagata and Victoria IBV lineages.

249 A handful of anti-viral drugs are also available for early treatment of illness, four of which
250 are approved in the U.S.: oseltamivir phosphate, zanamivir, peramivir, and baloxavir marboxil
251 (BXM) (28, 65). All are neuraminidase inhibitors and prevent release of viruses from cells, except
252 for BXM which acts on the PA protein to inhibit viral RNA synthesis (66). Two other anti-
253 influenza drugs, amantadine and rimantadine, have been used previously, but high rates of drug
254 resistance in seasonal strains have led to discontinuation (65, 67). During the 2020-2022 SARS-
255 CoV-2 pandemic, social-distancing measures, travel restrictions, and widespread use of masks
256 covering the mouth and nose are attributed to a dramatic decrease in the number of influenza virus
257 infections (68, 69).

258

259

Pandemic influenza viruses

Influenza pandemics occur when a novel strain is introduced into a naive human population that can sustain inter-human transmission. The spread of these viruses is not constrained by seasonality and is difficult to predict. Since 1900, there have been four influenza virus pandemics. The largest was the 1918-19 influenza pandemic, during which an H1N1 virus infected up to a third of the global population (15, 70). In 1957-1958, an H2N2 virus containing three genes of avian origin killed more than one million people around the globe. A decade later in 1968, an H3N2 influenza virus arose from H2N2 reassorting with an avian virus (15). While neuraminidase-specific immune protection in individuals exposed to the 1957 H2N2 virus likely dampened the severity of the pandemic, up to 4 million people are estimated to have died worldwide (71). Most recently, in 2009, the reassortant pdmH1N1 “swine” flu virus swept across the globe, largely impacting children and young adults (72, 73). After each pandemic waned, the causative virus continued to circulate in humans as a seasonal strain, replacing a previously-circulating lineage.

Intermittent zoonotic transmission occurs at human-animal interfaces, such as farms or live animal markets. Most of the time, these infections do not spread very far as the viruses are maladapted for sustained human transmission. Several characterized IAVs are, however, considered potential pandemic viruses, including H5N1, H7N9, and H9N2 influenza viruses, as small outbreaks have occurred in humans (15, 74, 75). H5N1 IAVs are responsible for many of these outbreaks, causing 864 cases and 456 deaths since 2003, and have been seen in short chains of human-human transmission (38, 76, 77). Small stockpiles of vaccines against defined potentially-pandemic IAVs as well as antivirals dedicated to future pandemics are kept in the U.S. in the event that one of these or a yet-undefined zoonotic IAVs adapts to mammalian spread.

283 ***Interhost transmission***

284 *Barriers*

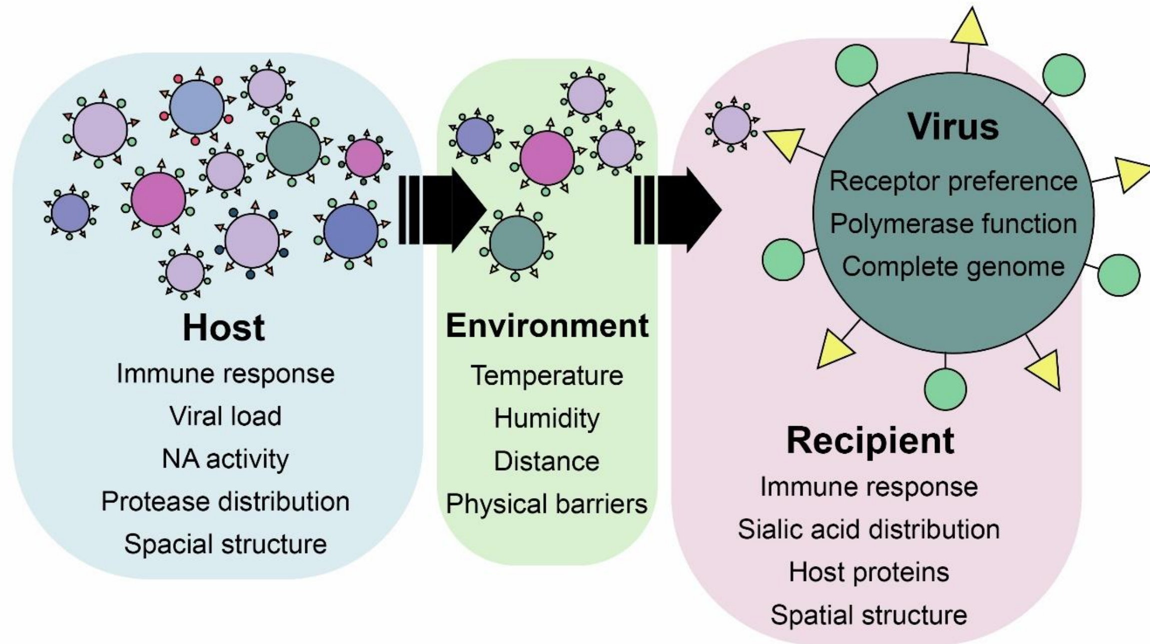

Fig. 3: Barriers to IAV spread. Some of the identified barriers determining whether a virus can spread from a source host to a recipient host (2-5). Bolded factors are discussed in the text. (Graphic: J. Shartouny)

285 The ability of IAVs to successfully transmit between individuals and complete a replicative
286 cycle is impacted by many factors within the original host, environment, and the potential recipient
287 host. Each barrier encountered decreases the overall genetic diversity of the influenza population,
288 known as genetic bottlenecking. The large population of viruses arising from a single infected host
289 are subjected to conditions that eliminate the infection potential of many of those viruses, leading
290 to a much smaller population that transmit to a new host to seed a new infection. Transmission
291 between individuals of different species encounters still more hurdles to successful replication.

292 Upon initial exposure to an IAV, host cell receptors dictate whether the virus can attach to
293 a cell to initiate entry. The linkage pattern of sialic acid to galactose on cellular glycoproteins and
294 the distribution of each sialic acid moiety on tissue surfaces in the body determine the tropism of

295 IAVs based on HA receptor compatibility (78). In many avian IAVs, HA preferentially binds
296 terminal sialic acids with α2,3 linkages, which occur mainly in the gastrointestinal tract of birds.
297 Human-adapted strains of IAV bind sialic acids linked via α2,6 to galactose, which are prevalent
298 in the upper respiratory tract in humans and leads to respiratory disease (79). Modification of
299 amino acids in and near the receptor binding site of HA can modify receptor preferences.

300 The lower respiratory tract in humans also contains α2,3 linked sialic acids, which might
301 account for zoonotic transmission seen in those who are often exposed to poultry, as repeated and
302 prolonged exposure presents many opportunities for a virus to travel deep enough into the lungs
303 despite attachments being rare (80). The lower respiratory tract of humans has a higher pH and
304 temperature than the upper tract which can also limit how far into the respiratory system a virus
305 can be viable, as avian and mammalian-adapted viruses differ by temperature and pH tolerance
306 (81). A study of an H5N1 influenza virus strain found that the virus could attach to pneumocytes
307 in the lower respiratory tract and to alveolar macrophages that could support viral dissemination
308 into non-respiratory tissues if this virus can penetrate into the lungs (80, 82).

309 The NA protein also plays a role in species specificity as it cleaves sialic acid moieties on
310 cell surfaces to release new viruses. Drifting of linkage specificity of N2 viruses from being
311 specifically α2,3 to also acting upon α2,6 was seen over 30 years after the 1968 pandemic and is
312 thought to be moving toward alignment with human-adapted viruses' HA linkage preferences (83).
313 A balance between the specificity of HA and enzymatic activity of NA must be met to allow viral
314 dissemination (84).

315 Once a virus has entered a cell, it interacts with host proteins to initiate and complete the
316 infection and replication process. Variations in protein forms and content between species can
317 impact infection. Though there are many, one host factor, ANP32A, has been identified to potently

318 impact influenza polymerase function (85). The avian form of this protein contains 33 amino acids
319 that are not included in the human form so avian-adapted viruses cannot efficiently interact with
320 the human ANP32A (81). A single amino acid change in PB2, E627K, can allow avian influenza
321 polymerases to interact with human ANP32A (86, 87). Transport of the viral RNPs and viral
322 proteins into the nucleus depends on host cell importin proteins to cross the nuclear membrane.
323 Nuclear localization signals located in the IAV NP and polymerase proteins interact preferentially
324 with different importins depending on the signal sequence and modification of specific residues,
325 for example NP 319 can allow avian viruses to interact with mammalian importins (88).

326 The proteins that make up the IAV polymerase heterotrimer, PB2, PB1, and PA, have also
327 been shown to contribute to species-specific infection barriers. The polymerase complex
328 transcribes viral mRNA and replicates the genome. Modification of the amino acid sequence of
329 the polymerase proteins can change IAV pathogenicity and host compatibility (74, 89). In all three
330 polymerase proteins, many single amino acid changes are related to better polymerase activity in
331 mammals (90). PB2 E627K, which is important in host protein interaction, is also thought to
332 regulate temperature sensitivity of the polymerase and allows more efficient replication at the
333 lower temperature of a mammalian upper respiratory tract versus the higher temperature of an
334 avian gastrointestinal tract (91). As with 627K, PB2 701N is seen in viruses isolated from humans
335 infected with H7N9 and may allow mammalian adaptation despite the presence of PB2 627E (86).
336 Similarly, research has identified residue changes in avian PB1 and PA that are thought to increase
337 pathogenicity in mammals (89, 92, 93).

338 H7N9 avian influenza viruses have caused several zoonotic outbreaks and have been
339 reported to spread in contact clusters between humans, though have not achieved sustained inter-
340 human transmission (94). This is worrisome from a pandemic surveillance standpoint, as just a

341 few mutations are seen to allow avian viruses to spread to humans and replicate. A study of
342 influenza A/Anhui/1/2013 (H7N9) virus (AH13) showed that it replicates to high titer in poultry
343 with minimal apparent disease, allowing accumulation of mutations and evolutionary potential
344 (95). AH13 could transmit to ferrets, who then passed it via direct contact and, to a limited extent
345 aerosols to other ferrets. Despite replication ability, there was much less diversity found within
346 ferrets than within the chickens and selection for mammal-adapted genotypes was not seen (95).
347 The genetic bottleneck in the transmission and infection of H7N9 from birds to mammals, which
348 could arise from any of the barriers encountered during the viral lifecycle as well as stochastic
349 barriers, reduced the chances of mammalian adaptation (95).
350
351 *Overcoming barriers*
352 Due to the numerous hurdles to establishing an infection, most cross-species transmission
353 events are non-productive. All IAV likely descended from avian viruses, however, and the
354 sustained circulation in mammalian and poultry populations as well as emergence of pandemic
355 influenza clearly shows that infections of new hosts occur. Genetic diversification via mutation
356 and reassortment gives the evolutionary potential for host range expansion. Avian influenza
357 viruses reassort with high frequency and, if passed to domestic animals like poultry or pigs
358 accumulate additional mutations and reassort with IAVs circulating in those populations, as was
359 seen in the 2009 pandemic (96).
360 Reassortment relies on multiple viral infection of a single cell to allow gene segments from
361 two or more parental viruses to be packaged into progeny. Coinfections also impact infections in
362 other ways, through collective interactions. Viral populations consist of many related but
363 genotypically varied particles (97). If these are transmitted together, a single cell can receive more

364 than one viral genome, either heterologous or homologous. Collective infectious units increase the
365 multiplicity of infection of a cell and are seen in a number of viruses with different modes of
366 transmission between cells (98).

367 Genomic complementation is one consequence of multiple infection. Failure to replicate
368 one or more IAV genome segments could lead to an abortive single infection, however multiple
369 available genomes could provide the missing components to the insufficient virus. Segments
370 appear in a population at an intermediate frequency (99, 100). If a semi-infectious particle enters
371 a cell at the same time as another virus that possesses the missing segment, the virus can share the
372 resources of the other to complete a replicative cycle(101). Cooperation has also been noted
373 between similar H3N2 influenza genotypes, perhaps due to complementary entry and exit
374 capabilities (102).

375 Several viruses besides IAV show collective interactions that impact their pathogenicity in
376 various ways. Vesicles containing multiple viruses released from a single cell have been described
377 in poliovirus, rotavirus, and norovirus (103). These aggregates facilitate efficient movement of
378 multiple genomes at once and have increased infectivity over single viruses (104, 105). A measles
379 virus that delivers two heterologous genomes to a single cell was found to have enhanced fusion
380 capabilities versus either genotype individually (106). In HIV-1 infections, delivery of multiple
381 genomes to a cell has been seen to negatively affect antiviral effectiveness, as it establishes a
382 reservoir more quickly than singular genomes (103, 104).

383

384 ***Reliance on multiple infection***

385 Coinfections of influenza viruses occur with high frequency in animal models (105, 107,
386 108). Complementation of semi-infectious particles is seen at high levels in guinea pigs, indicating

387 the delivery of multiple
388 genomes to a cell (109).
389 Spatial structure of the
390 host tissue and tropism of
391 the transmitted viruses are
392 determinants of
393 coinfection frequencies,
394 implying that more similar
395 viruses are more likely to
396 coinfect (99, 107).
397 Previous experiments have
398 shown that homologous
399 coinfections can accelerate
400 viral replication and
401 transcription (1) and further
402 investigation of the consequences of homologous multiple infection is needed.

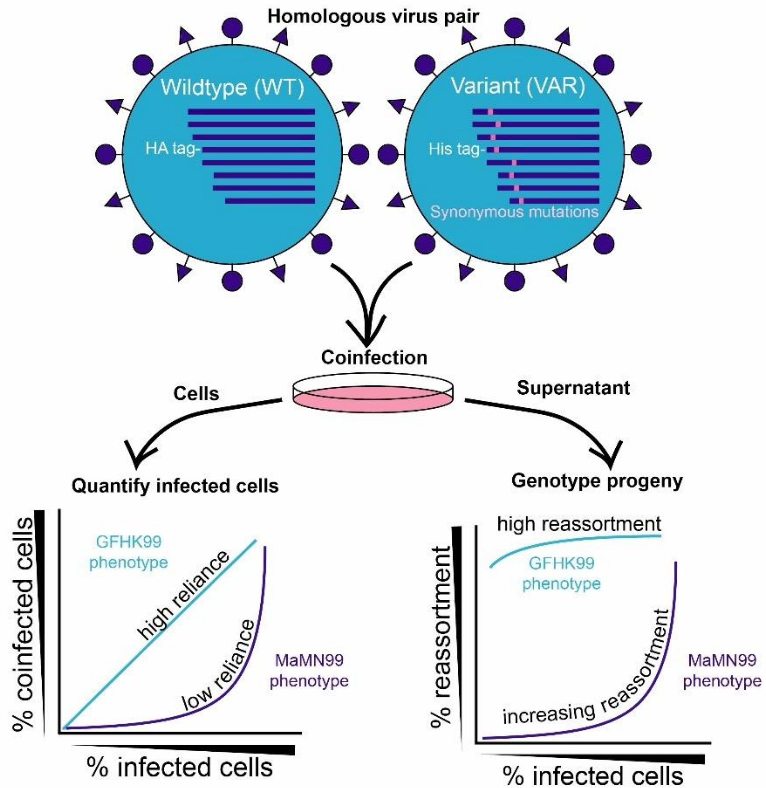

Fig. 4: The Lowen Lab multiple infection model. The WT/VAR system for evaluating multiple infection uses homologous virus pairs to infect cells. Infected cells, coinfected cells, and reassortant progeny can be quantified. (Graphic: J. Shartouny)

403 The dynamics of homologous coinfections can be investigated using a model system that
404 differentiates between two essentially identical viruses that each have a unique epitope tag.
405 Developed by the Lowen Lab, the WT/VAR multiple infection model allows in vitro quantification
406 of singly- and co-infected cells using a wildtype (WT) virus alongside a virus that contains a
407 synonymous mutation in each segment called the variant (VAR) virus (1). Frequencies of
408 coinfected cells within a population of cells can then be analyzed for different strains at varied
409 MOIs in multiple cell types. If frequencies of coinfected cells within an infected population follow

410 a Poisson distribution, which assumes each infection event is random, independent of other
411 infections, and evenly distributed over the population of cells, there will be a mixture of singly-
412 and multiply-infected cells. In this scenario, frequencies of coinfection would increase with
413 increasing MOI (99). An assortment of single-cycle IAV WT/VAR coinfections determined
414 frequencies of coinfected cells does increase with MOI (110). In every virus tested, however,
415 coinfections increased more rapidly than would be expected if infection proceeded irrespective of
416 the number of input genomes. This indicates that a moderate reliance on multiple infection impacts
417 the infection phenotype of each virus (Fig. 4).

418 A particularly interesting observation
419 from these data was that the avian virus
420 influenza A/guinea fowl/Hong Kong/WF10/99
421 (H9N2) virus (GFHK99) showed an
422 intermediate reliance on multiple infection
423 when infecting avian cells (DF-1 cells), on par
424 with other tested viruses, but when used to
425 infect mammalian cells (MDCK cells), the
426 frequency of infected cells and coinfected cells
427 becomes the same, giving a linear relationship.
428 This indicates that most detectable productive
429 cells are multiply infected. This high reliance
430 on multiple infection seen in mammalian cells
431 but not avian cells cannot be attributed only to
432 incomplete genomes, according to previous

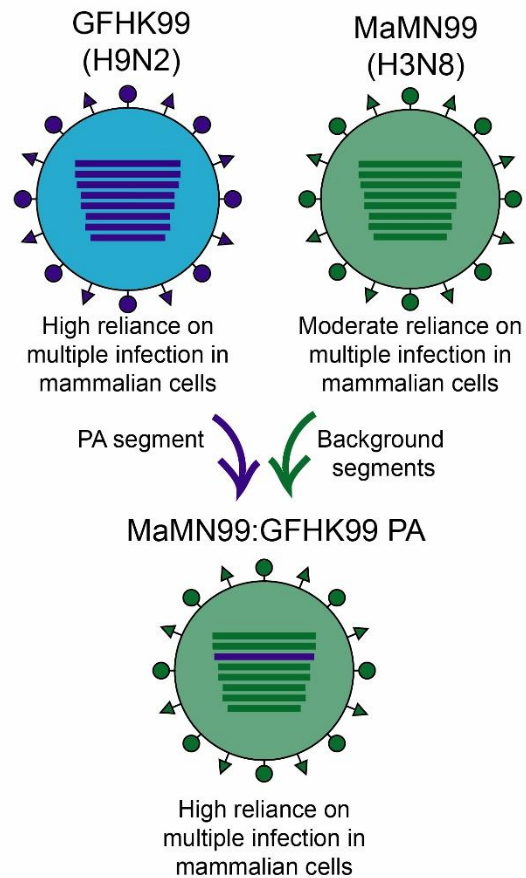

Fig. 5: GFHK99 high reliance is linked to the PA gene segment. Data were reported previously in Phipps, et al. 2021 (1). (Graphic: J. Shartouny)

research (111), so there are other viral or host factors determining the extent to which GFHK99 needs multiple infection.

Previous work in the Lowen lab sought to define which viral gene segment influenced this high reliance seen by GFHK99 through comparisons with another virus for which host-specific reliance on multiple infection is not seen, A/mallard/Minnesota/199106/99 (H3N8) virus (MaMN99) (89). Through systematic segment replacement of the MaMN99 genome with singular segments from GFHK99 using a homologous coinfection model system, the PA gene segment was identified as a major determinant of the high reliance seen in GFHK99 (Fig. 5). Additional experiments with an IAV that is fully-reliant on multiple infection within a compatible host showed that it could replicate in an animal host, but not transmit efficiently to contact animals and infection is mediated by single viruses (99). In the case of an avian virus infecting mammalian cells, however, it seems that a single genome is insufficient for productive infection, which has implications for zoonotic transmission of IAVs requiring simultaneous delivery of multiple viruses.

H9N2 viruses like GFHK99 are also known to reassort with other co-circulating IAVs (2). Reassortants can display traits such as differential receptor glycan binding that are more conducive to mammalian infection (3, 5, 89). A virus encountering a new host might overcome host restriction through homologous multiple infection, allowing it to reproduce and intermingle with viruses adapted to that particular species and increasing the chances that a progeny IAV can transmit between a novel host.

Dissertation aims

Continuing investigation of GFHK99 dependence on multiple infection, this dissertation aimed to pinpoint more specifically the viral determinant of the reliance phenotype. The PA gene segment was previously identified as integral to multiple infection reliance and was thus examined at a functional domain level, finding that the endonuclease region of PA is linked to multiple infection. Upon finer examination of the PA endonuclease, amino acid replacements identified PA 26 as the major determinant of multiple infection reliance. GFHK99 PA K26E mutant viruses display increased frequencies of productive singly-infected cells. PA 26K was found to reduce endonuclease activity, leading to inefficient viral transcription. Increasing the number of genomes in a cell, however, allows the virus to overcome this barrier to infection.

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465

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Chapter 2

Beneficial effects of cellular coinfection resolve inefficiency in influenza A virus transcription

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Abstract

For diverse viruses, cellular infection with single vs. multiple virions can yield distinct biological outcomes. We previously found that influenza A/guinea fowl/Hong Kong/WF10/99 (H9N2) virus (GFHK99) displays a particularly high reliance on multiple infection in mammalian cells. Here, we sought to uncover the viral processes underlying this phenotype. We found that the need for multiple infection maps amino acid 26K of the viral PA protein. PA 26K suppresses endonuclease activity and viral transcription, specifically within cells infected at low multiplicity. In the context of the higher functioning PA 26E, inhibition of PA using baloxavir acid augments reliance on multiple infection. Together, these data suggest a model in which sub-optimal activity of the GFHK99 endonuclease results in inefficient priming of viral transcription, an insufficiency which can be overcome with the introduction of additional viral templates to the cell. These findings offer rare mechanistic insight into the benefits of viral collective dispersal.

756 **Introduction**

757 Influenza A viruses (IAVs) impose a substantial burden on public health and agriculture
758 each year. In humans, IAVs circulate seasonally, causing several million illnesses per year
759 globally, and have caused four pandemics since 1918 (1). Fundamental understanding of IAV
760 biology is critical to the design of optimal strategies for prevention and control of influenza.

761 As has been seen for diverse viral species (2-6) interactions between homologous,
762 coinfecting, IAVs can be highly biologically significant (7-9). While most single infections are
763 abortive, delivery of multiple viral genomes to a cell strongly increases the likelihood of
764 productive infection and can augment both replication rate and yield (7, 8, 10). In other words,
765 the virus-virus interactions that play out during cellular coinfection are typically beneficial and
766 often required for productive infection. Of note, this feature of IAV biology strongly increases
767 the frequency of reassortment, an important source of viral genetic diversity (11).

768 IAV reliance on multiple infection appears to be particularly acute under conditions that
769 are unfavorable for viral replication, such as in a new host species (8). For a G1-lineage strain,
770 influenza A/guinea fowl/Hong Kong/WF10/99 (H9N2) virus (GFHK99, also referred to as
771 WF10), we found that coinfection with multiple homologous viruses was essential for robust
772 replication in mammalian cells, but not avian cells. While every IAV tested thus far has shown
773 some reliance on multiple infection, the strong host-specific reliance of GFHK99 was not
774 apparent for influenza A/mallard/Minnesota/199106/99 (H3N8) virus (MaMN99). Using gene
775 segment reassortments between these two IAVs, we found that the PA segment drives the high
776 reliance of GFHK99 virus on multiple infection. However, the mechanistic basis for this genetic
777 association remained unclear.

778 The PA gene segment encodes two proteins: PA and PA-X. PA is one of three protein
779 subunits that comprise the viral RNA-dependent RNA polymerase, along with PB2 and PB1.
780 The N-terminus of PA contains an endonuclease that cleaves cellular mRNAs 10-20 bases
781 downstream of the 5' cap, in a process termed cap-snatching (12, 13). The resultant capped
782 primer is required for mRNA synthesis by the viral polymerase (14). PA-X, discovered in the
783 past decade, is produced by ribosomal frame-shifting during translation of the PA mRNA (15).
784 The N-terminal 191 amino acids of PA-X are identical to those of PA, while the C-terminal 41 or
785 61 amino acids are unique (16). PA-X has been shown to contribute to the shutoff of host protein
786 synthesis (17).

787 In this work, we sought to elucidate the mechanistic drivers of the GFHK99 strain's high
788 reliance on multiple infection, tied to the PA gene segment. Our data demonstrate that the
789 phenotype maps to residue 26K within the PA endonuclease. This amino acid lowers
790 endonuclease activity, leading to inefficient viral transcription in cells infected at low
791 multiplicity of infection (MOI). Conditions conducive to cellular coinfection allow robust
792 transcription by a PA 26K virus in mammalian cells and support enhanced progeny production.
793 Treatment of infected cells with a PA endonuclease inhibitor, baloxovir acid, leads to a similar
794 reliance on multiple infection. These data suggest that cellular coinfection is beneficial because
795 the delivery of multiple copies of the eight viral ribonucleoproteins (vRNPs) to the cell increases
796 the frequency of successful transcription events, allowing infection to be initiated efficiently
797 despite sub-optimal conditions.

798 **Results**

799 *Reliance on multiple infection of GFHK99 is linked to the endonuclease region of PA*

800 To determine which region of the PA gene segment contributed to the high reliance on
801 multiple infection seen in GFHK99, chimeric PA gene segments were created using GFHK99
802 PA and MaMN99 PA. The segment was divided into three regions roughly corresponding to the

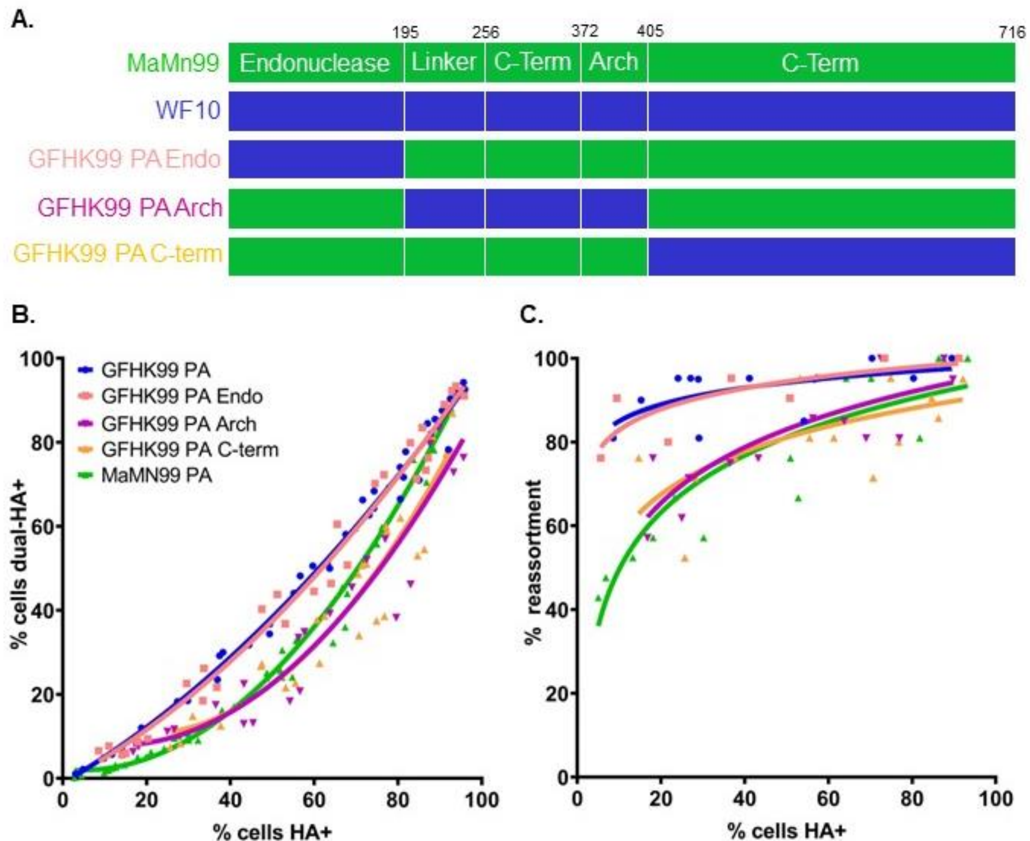

Fig 1. Replacement of the MaMN99 endonuclease region with that of GFHK99 PA increases reliance on multiple infection. (A) Chimeric PA gene segments that introduce regions of the GFHK99 PA into the MaMN99 PA: the endonuclease (GFHK99 Endo), the middle region (GFHK99 Arch), and the C-terminal domain (GFHK99 C-term). These segments were incorporated into the MaMN99 background. (B) The relationship between the percentage of the cell population coinfected with WT and VAR (dual HA+) and the percentage that is infected with either or both viruses (HA^+). Data plotted is from three independent experiments. (C) The percentage of progeny viruses with any reassortant genotype (% reassortment) is plotted against the percentage of cells expressing hemagglutinin (HA^+). The PA segment genotype of WT and VAR viruses is indicated in the legend. All viruses carried the remaining seven segments from MaMN99 virus. Data shown for each virus are derived from two independent experiments.

803 domains of the PA protein (Fig 1A). Each chimeric segment and the full-length GFHK99 PA
804 were incorporated into the MaMN99 background. To allow virus-virus interactions to be

805 monitored, homologous infection pairs, termed wildtype (WT) and variant (VAR) viruses, were
806 produced for each PA genotype. VAR viruses contained a synonymous mutation in each of the
807 eight gene segments to allow differentiation from WT segments in molecular assays. In addition,
808 WT and VAR virus HA proteins were differentially modified with epitope tags to allow
809 quantification of cellular infection at a protein level (8).

810 Using flow cytometry, the frequency of cellular coinfection between WT and VAR
811 viruses was evaluated. Since this assay detects viral protein, it measures the extent to which viral
812 gene expression relies on multiple infection. MDCK cells were coinfected with homologous WT
813 and VAR pairs and, to enable quantitative analysis, infections were limited to a single cycle such
814 that progeny viruses cannot be propagated onward. The relationship between the percentage of
815 cells that stained with one or both epitope tags (total HA^+) and the percentage of cells staining
816 with both tags (dual-HA^+) was evaluated (Fig 1B). As seen previously, MaMN99 WT and VAR
817 viruses produced distinct populations of singly-infected cells and coinfected cells, with the
818 frequency of coinfection increasing with total infection levels. Conversely, for the GFHK99 PA
819 in a MaMN99 background (GFHK99 PA virus), nearly all infected cells were coinfected with
820 both WT and VAR. Thus, a linear relationship was seen between dual-HA^+ and total HA^+, with a
821 slope of 1.02 (95% C.I. 0.979 to 1.07, R^2= 0.981). Both the GFHK99 Arch PA and GFHK99 C-
822 term PA viruses showed similar infection patterns to that of MaMN99 virus. In contrast, the
823 GFHK99 Endo PA virus displayed a linear relationship between dual-HA^+ and total HA^+, like
824 the GFHK99 PA virus. The slope obtained from a linear regression of GFHK99 PA Endo was
825 comp arable to that of GFHK99 PA at 1.03 (95% C.I. 0.959 to 1.10, R^2= 0.971).

826 The prevalence of reassortant viruses within the progeny virus population was then
827 determined. Since only cells coinfected with WT and VAR viruses can produce reassortants, this

828 assay gives an indication of the relative productivity of singly- and multiply-infected cells.
829 Reassortants were identified by deriving clonal isolates from the progeny population and then
830 genotyping each segment therein. The frequency of reassortants within MaMN99 progeny virus
831 populations was low at lower infection levels and increased as the percentage of HA^+ cells
832 increased. By comparison, GFHK99 PA virus coinfections resulted in high frequencies of
833 reassortment even at low levels of infection, signifying that most of the progeny viruses were
834 produced in cells infected with both WT and VAR (Fig 1C). WT-VAR coinfections with
835 GFHK99 Arch and GFHK99 C-term viruses exhibited similar reassortment outcomes as seen
836 with MaMN99 virus. Conversely, GFHK99 Endo PA coinfections displayed high percentages of
837 reassortant progeny, on par with those seen for GFHK99 PA. The high frequencies of reassortant
838 progeny and high levels of dual HA positivity observed in coinfections with GFHK99 Endo PA
839 viruses indicate that the endonuclease region of GFHK99 PA confers a high reliance on multiple
840 infection.

841 *Disruption of PA-X does not alter reliance on multiple infection*

842 The endonuclease domain is shared by the PA and PA-X proteins. To determine whether
843 PA-X was the driving force behind high reliance on multiple infection, viruses were created in
844 which the PA-X reading frame was disrupted (17). Because PA-X is difficult to detect by
845 western blotting, the effectiveness of this disruption was verified at a functional level. Using
846 plasmid transfection, the full length MaMN99 PA gene segment with or without mutations to
847 PA-X was introduced into cells together with a *Renilla* luciferase reporter construct. The wild
848 type PA construct strongly suppressed the reporter signal relative to that seen with the ΔPA-X

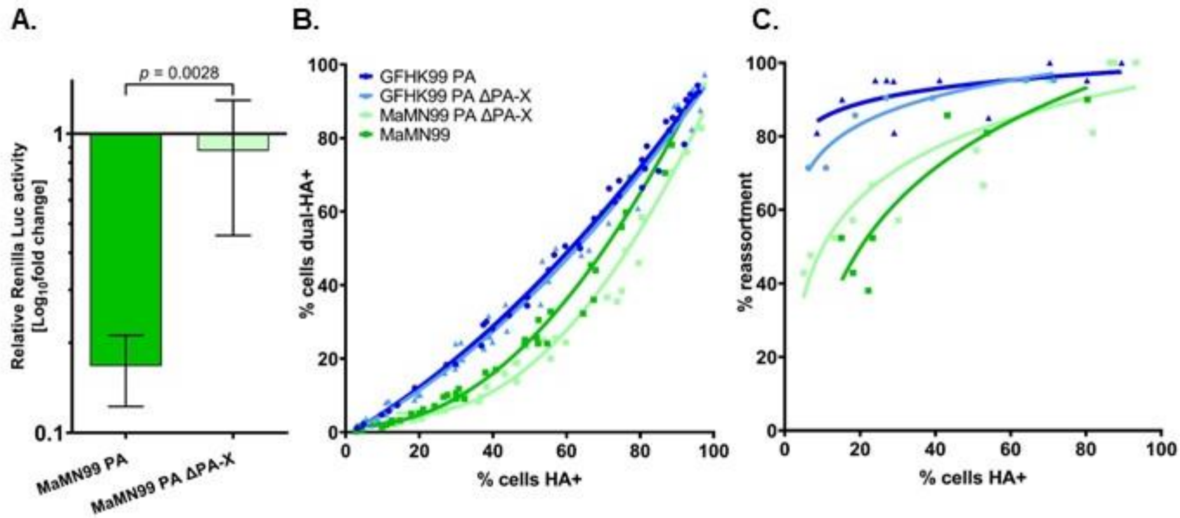

Fig 2. Disrupting PA-X does not alter reliance on multiple infection phenotype of GFHK99 and MaMN99 viruses. (A) Relative luciferase activity of cells cotransfected with a *Renilla* luciferase expression plasmid and either MaMN99 PA or MaMN99 PA ΔPA-X expression plasmids. Values represent means ± SDs of three replicates. Statistical significance was analyzed by Unpaired t-test. (B) The relationship between cells dually-infected with WT and VAR MaMN99 ΔPA-X or GFHK99 PA ΔPA-X and cells infected with WT, VAR, or both. Data plotted are from two independent experiments. (C) The percentage of progeny viruses with any reassortant genotype is plotted against the percentage of cells expressing hemagglutinin (HA). The PA segment genotype of WT and VAR viruses is indicated in the legend. All viruses carried the remaining seven segments from MaMN99 virus. Data shown for each virus are derived from two independent experiments. Results for GFHK99 PA and MaMN99 viruses are reproduced from Figure 1 for comparison.

849 construct, consistent with the host shut-off activity of PA-X (17) and confirming the

850 effectiveness of the mutations introduced (Fig 2A).

851 WT and VAR homologous pairs of these viruses were used to coinfect MDCK cells and

852 the frequencies of coinfected cells and reassortant viruses were examined across a range of MOIs

853 (Fig 2). By both measures, MaMN99 ΔPA-X and GFHK99 PA ΔPA-X viruses displayed similar

854 coinfection reliance phenotypes to their respective parental strain. Since expression of PA-X did

855 not modulate the extent of reliance on multiple infection in either strain, PA-X does not appear to

856 be a major driver of the high reliance phenotype displayed by GFHK99.

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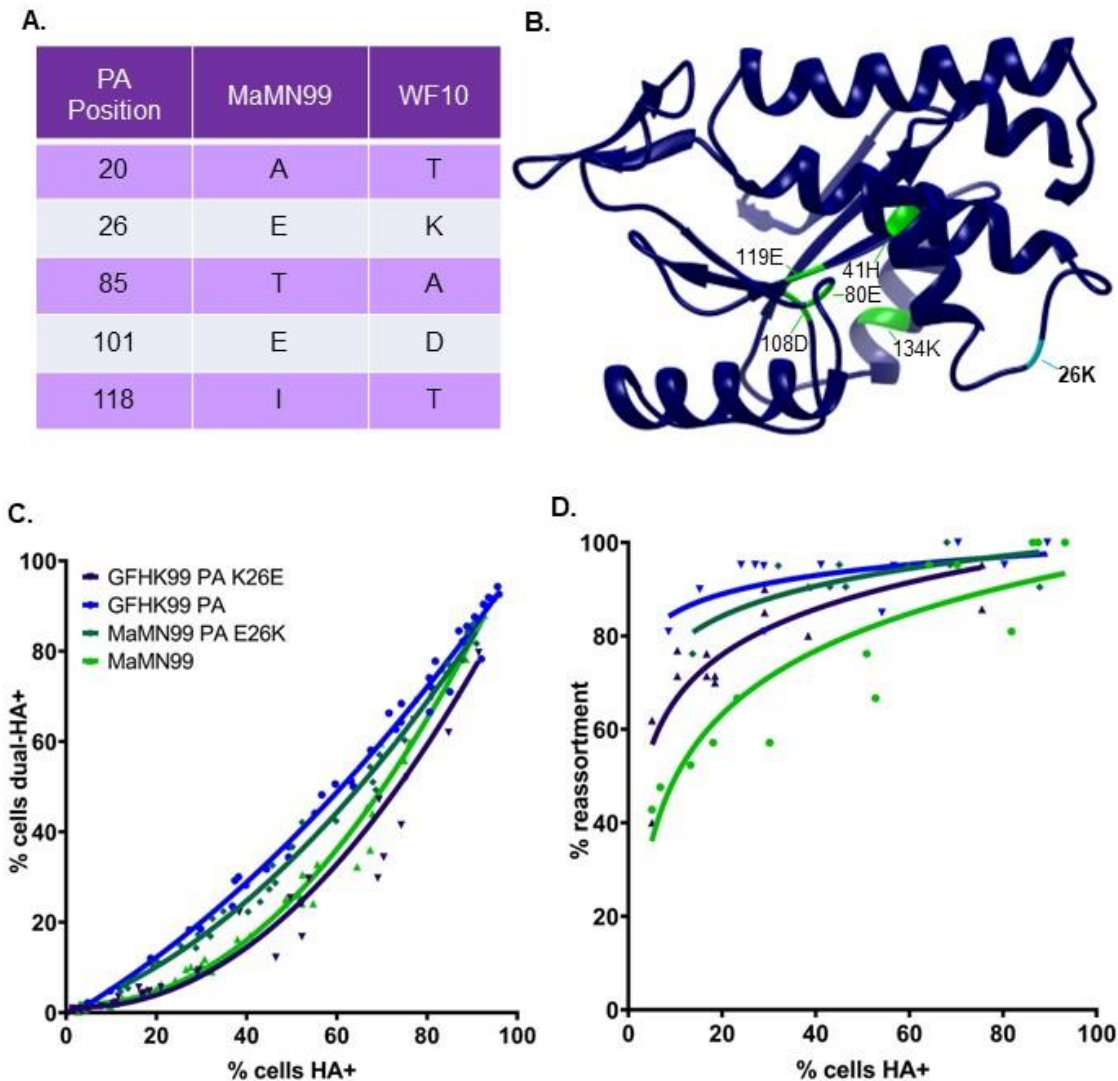

PA Position	MaMN99	WF10
20	A	T
26	E	K
85	T	A
101	E	D
118	I	T

Fig 3. PA 26K is associated with a higher reliance on multiple infection than PA 26E. (A) An amino acid alignment of MaMN99 and GFHK99 PA endonuclease regions shows five amino acid differences. (B) The endonuclease domain of GFHK99 PA with the active site residues labeled in green and PA 26K labeled in cyan. (C) The relationship between the percentage of the cell population infected with both WT and VAR of MaMN99 PA E26K or GFHK99 PA K26E (dual HA+) and the percentage that is infected with either or both viruses (HA+). Data plotted is from three independent experiments. (D) The percentage of reassortant progeny derived from MaMN99 PA E26K and GFHK99 PA K26E from two independent experiments is plotted against the total percentage of cells expressing HA.

858 *Reliance on multiple infection is dependent on PA 26 in the endonuclease region.*

859 An alignment of the PA endonuclease regions of MaMN99 and GFHK99 showed five

860 coding differences at PA amino acids 20, 26, 85, 101, and 118 (Fig 3A). Owing to the charge

861 difference at PA 26, where MaMN99 has a glutamic acid (E) and GFHK99 has a lysine (K), we
862 focused on this position, which is located proximal to the active site of the endonuclease region
863 (Fig 3B). Reciprocal mutants were generated in the MaMN99 and GFHK99 PA segments and
864 each was incorporated into the MaMN99 background. WT and VAR homologous pairs of
865 MaMN99 PA E26K and MaMN99: GFHK99 PA K26E (GFHK99 PA K26E) viruses were used
866 in coinfections.

867 The results showed that swapping the amino acid at PA 26 swapped the phenotypes
868 displayed by MaMN99 and GFHK99 PA. Introduction of K26E within the GFHK99 PA
869 decreased frequencies of dually HA+ cells, while introduction of E26K into the MaMN99 PA
870 had the opposite effect (Fig 3C). In line with the coinfection results, GFHK99 PA K26E virus
871 yielded fewer reassortants than GFHK99 PA virus, while MaMN99 PA E26K infection progeny
872 were predominantly reassortant (Fig 3D). Thus, in both PA backgrounds, PA 26K was associated
873 with higher reassortment than PA 26E. Taken together, the data show that viral gene expression
874 and progeny production were focused within coinfected cells to a greater extent for viruses
875 encoding PA 26K compared to those encoding PA 26E.

876 *Endonuclease activity and transcript production are suppressed by PA 26K*

877 We reasoned that the high reliance on multiple infection resulting from PA 26K might be
878 a result of reduced PA protein levels in infected cells or impeded functionality of the PA protein.
879 To evaluate the first possibility, PA protein levels in cells infected with GFHK99 PA or
880 GFHK99 PA K26E viruses were compared by western blotting (Fig 4A). GFHK99 PA virus did
881 not display less PA protein than GFHK99 PA K26E virus, indicating that PA 26K did not reduce
882 the accumulation of PA during infection.

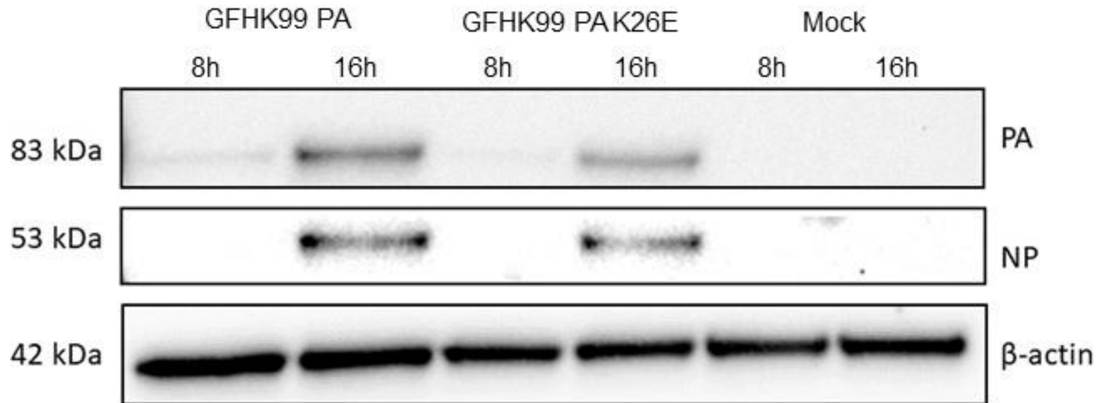

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Fig 4. Levels of PA protein are not decreased by PA 26K. Western blot of cell lysates collected at 8 or 16 h post-infection with GFHK99 PA virus, GFHK99 PA K26E virus, or mock infected. Blot was probed for PA, NA, and β-actin.

884 To evaluate viral endonuclease activity of MaMN99 and GFHK99 PA, we assayed the extent to
885 which the corresponding PA-X proteins disrupted expression of *Renilla* luciferase in transfected
886 cells. This approach was used because PA and PA-X carry the same endonuclease domain, but
887 PA-X activity is more readily monitored in a cell-based assay. As expected, based on the
888 mRNA-degrading function of PA-X, the activity of *Renilla* luciferase gradually decreased with
889 an increase in the amount of PA-X plasmid introduced into cells (Fig 5A). Of note, the effect
890 was markedly weaker with the GFHK99 PA-X than the MaMN99 PA-X, indicating that the
891 GFHK99 PA-X has lower endonuclease activity. Next, E26K or K26E variants of MaMN99 and
892 GFHK99 PA-X, respectively, were tested and PA-X expression of each was verified by Western
893 blot. The E26K mutation in MaMN99 PA-X reduced PA-X activity, showing less reduction of
894 *Renilla* luciferase activity than the wild-type (Fig 5B, 5D). Conversely, the K26E mutation in
895 GFHK99 PA-X enhanced PA-X activity (Fig 5C, 5E). Thus, position 26 within the viral
896 endonuclease modulates its enzymatic activity. We further measured the effect of the E26K
897 mutation in MaMN99 PA on viral transcription during infection by measuring the accumulation

898 of viral mRNA under low and high MOI conditions in MDCK cells. At a low MOI, markedly
899 less mRNA was produced in MaMN99-PA-E26K infection compared to MaMN99 infection (Fig
900 5F). However, at a high MOI, mRNA accumulated at a similar rate for MaMN99 and MaMN99-
901 PA-E26K viruses (Fig 5H). These data suggest that low endonuclease activity of PA carrying the
902 26K polymorphism suppresses viral transcription at a low MOI but is compensated by multiple
903 infection. Measurement of viral genomic RNA (vRNA) in the same cells (Fig 5G, 5I) revealed
904 similar patterns, indicating that the effects of PA 26K and multiple infection are also borne out at
905 the level of viral genome replication, most likely as a downstream consequence of the effects on
906 viral transcription.

907 *Inhibition of endonuclease activity increases reliance on multiple infection*

908 We postulated that inhibiting the PA endonuclease cap-snatching function would enforce
909 an increased reliance on cellular coinfection for productive replication. The drug baloxavir
910 marboxil (Xofluza) targets cap-snatching by chelating the ions in the active site of the PA protein
911 and baloxavir acid (BXA), the active form of the drug, is available for use in cell culture.
912 MaMN99 WT and VAR virus coinfections were treated with an intermediate dose of BXA
913 designed to handicap but not wholly abolish infection (Fig 6A). The frequency of reassortant
914 progeny resulting from these infections increased across the range of MOIs tested, indicating that
915 nearly all progeny arose from coinfected cells under BXA treatment. This outcome is in stark
916 contrast to that seen in mock-treated MaMN99 infections, where frequencies of reassortment
917 suggest that appreciable levels of virus emanate from singly infected cells.

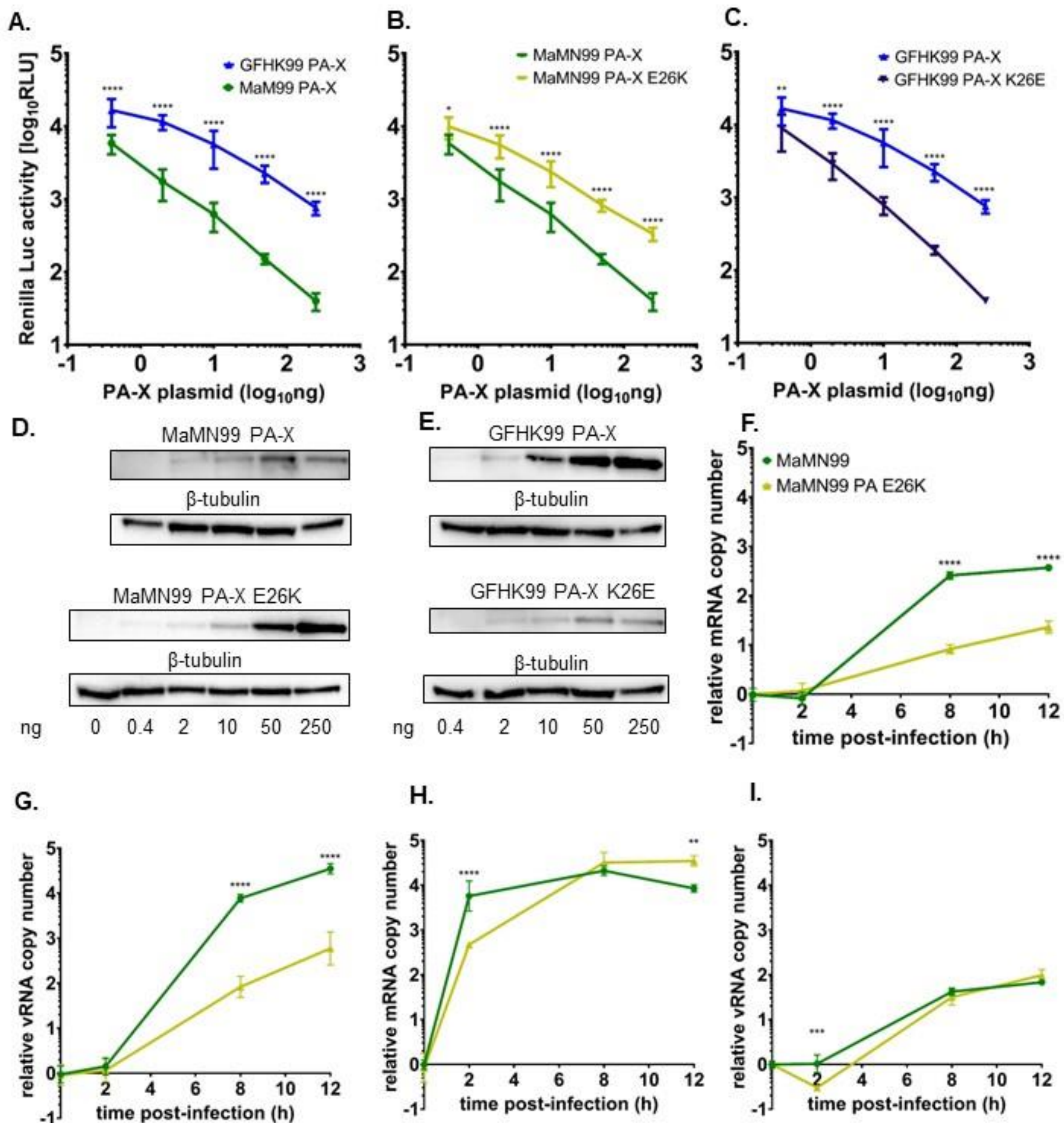

Fig 5. PA 26K confers lower endonuclease activity than PA 26E. (A-C) Renilla luciferase expression, plots include three independent experiments, compared via 2-way ANOVA. ****$p < 0.0001$, *p=0.0201, **p=0.0017. A. MaMN99 PA-X and GFHK99 PA-X. (B) GFHK99 PA-X and GFHK99 PA-X K26E. (C) MaMN99 PA-X and MaMN99 PA-X E26K. (D-E) Western blots show expressed PA-X and β-tubulin at each concentration of plasmid (ng). (F-I) mRNA (F, H) and vRNA (G, I) were measured at 0, 2, 8, and 12 hpi in a low MOI infection (F,G) and a high MOI infection (H, I). vRNA and mRNA levels were compared via 2-way ANOVA.

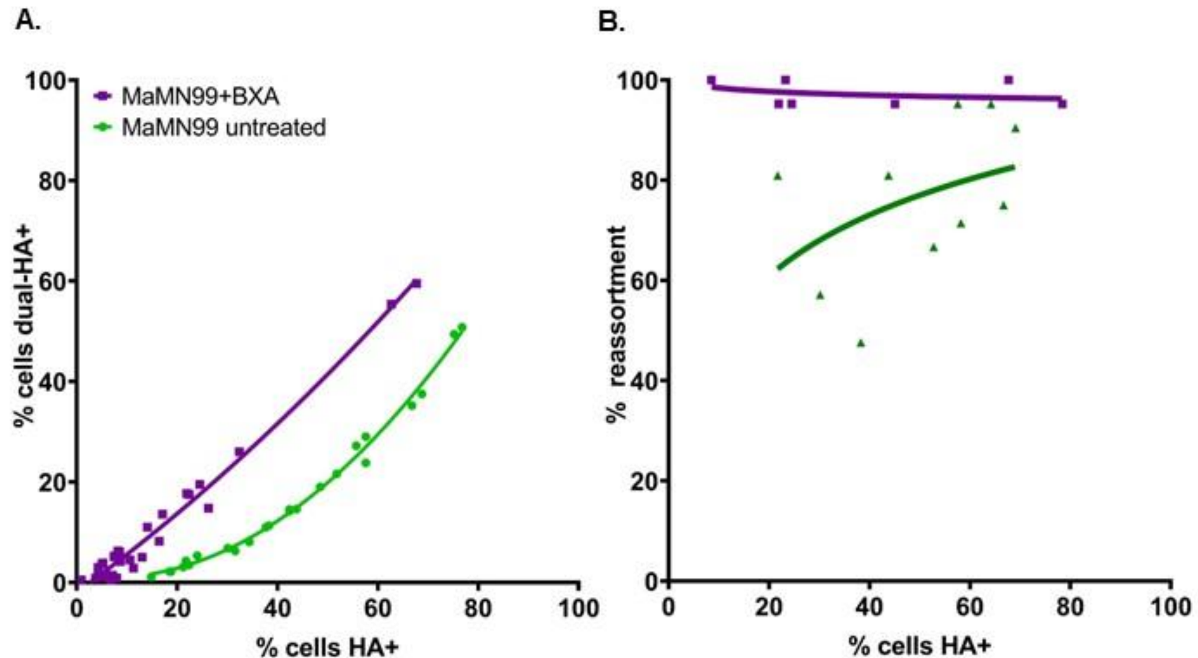

Fig 6: Inhibition of cap-snatching increases reassortment. (A) The MaMN99 PA endonuclease was inhibited using baloxavir acid (BXA) during a coinfection with homologous WT and VAR viruses and the relationship between percent cells HA+ and cells dual-HA+ was determined for two independent experiments. (B) The percentage of reassortant progeny resulting from MaMN99 PA +BXA and mock treated was compared to the total percentage of infected cells.

919 **Discussion**

920 Although cellular coinfection can strongly impact infection outcomes in many virus-host
921 systems, the mechanistic basis for these effects is generally poorly understood. Here we sought
922 to address this deficiency by focusing on GFHK99, an IAV that shows extremely high reliance
923 on cellular coinfection. We identified a polymorphism at position 26 within the PA endonuclease
924 domain as the driver of high reliance on multiple infection. Functionally, PA 26K reduces the
925 activity of the endonuclease enzyme, lowering the efficiency of viral transcription. Importantly,
926 however, under high MOI conditions, the inefficiency of transcription is overcome. Consistent
927 with these results, BXA-imposed inhibition of the PA endonuclease leads to a focusing of viral

928 replication within cells that are
929 multiply-infected. Our data
930 suggest a model in which the
931 delivery of many viral genomes
932 to the cell compensates for
933 inefficient cap-snatching by
934 providing greater opportunity for
935 that process to unfold (Fig. 7).
936 The result is a positive density
937 dependence, in which per capita
938 viral reproduction increases as
939 more viruses infect the same cell.

940 To successfully propagate
941 its genome, an infecting virus
942 must complete a number of
943 discrete steps of the early life
944 cycle, many of which are the targets of cellular defense mechanisms. For IAV, each barrier must
945 be overcome by eight unique vRNP complexes. This process appears to be prone to failure, such
946 that the likelihood of a single IAV establishing an infection is extremely low (10, 18). Consistent
947 with low rates of productive infection, IAV infected cells typically support expression from and
948 replication of fewer than eight segments (7, 10). In the case of GFHK99 virus, incomplete viral
949 genomes were detected with a frequency of approximately 90% (8). While high, this frequency is
950 not sufficient to account for the extremely high reliance on multiple infection exhibited by this

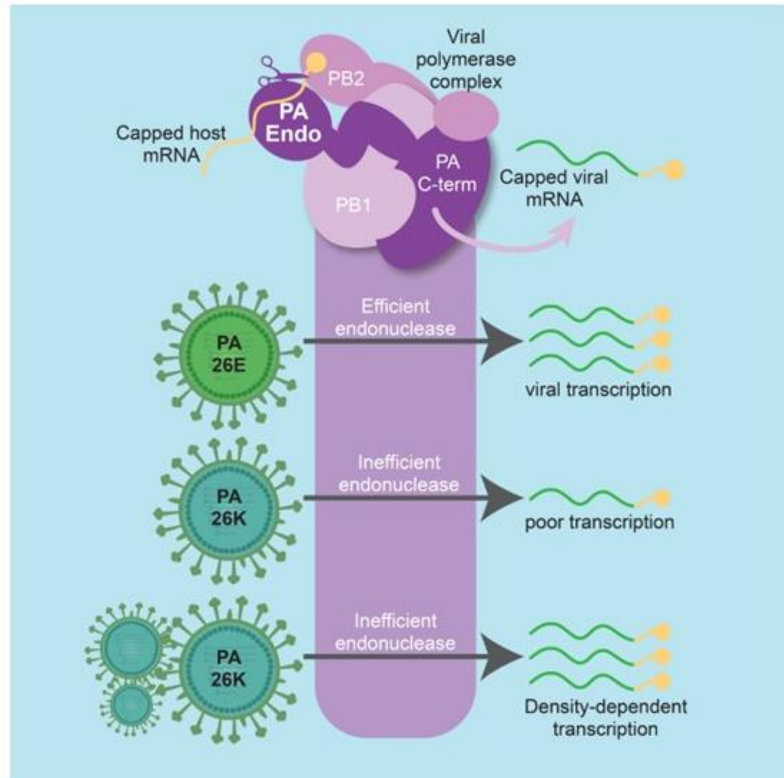

Fig 7. Multiple infection allows a virus with an inefficient PA endonuclease to replicate. The PA protein, located in the polymerase complex, cleaves host mRNA caps to allow the viral polymerase to transcribe viral mRNAs. Our data show that a virus containing PA 26E can successfully transcribe viral mRNA while a virus containing PA 26K cannot do this as efficiently. Multiple infection, however, allows a virus with PA 26K to overcome this hurdle.

951 strain in mammalian systems (8). The data reported here indicate that this additional reliance
952 stems from a deficiency in viral cap-snatching, the process by which the IAV polymerase
953 acquires capped RNA oligomers to prime transcription (13). With multiple viral genomes
954 infecting together, the larger number of templates (and associated polymerases)
955 available for primary transcription appears to enable levels of mRNA synthesis sufficient to
956 sustain robust infection.

957 Our previous work revealed that the extent to which GFHK99 virus relies on multiple
958 infection for productive infection is strongly dependent on host species: in avian systems, the
959 need for multiple infection was greatly diminished (8). Since we focused our studies herein on
960 mammalian systems, it is not clear whether the GFHK99 PA enzyme is more active in avian
961 cells or whether this avian virus is simply less sensitive to the cap-snatching deficiency in a host
962 environment to which it is well adapted. This is a question to pursue in future work.

963 In using BXA treatment to substantiate the link between deficient cap-snatching and the
964 need for many incoming viral genomes, we show that - within multiply-infected cells - viral
965 replication can proceed under treatment with the active form of an FDA-approved antiviral drug.
966 While potentially consequential for clinical use of baloxavir marboxil and the development of
967 resistance (19, 20), it is important to consider that increasing the dose of BXA in our
968 experimental system led to a complete block of infection. In fact, our data more broadly suggest
969 that the targeting of viral cap-snatching and other steps of the viral life cycle that precede
970 genome replication are likely to be efficient therapeutic strategies: this approach is expected to
971 increase the frequency of abortive infections, potentially pushing the rate of productive
972 infections within a host below a threshold needed to sustain viral propagation.

973 The critical mutation identified, PA 26K, occurs rarely in sequenced influenza strains,
974 including those of the G1 lineage of H9N2 viruses (21). Deleterious effects of PA 26K in
975 mammalian and, to a lesser extent, avian systems were identified in previous work using the
976 GFHK99 strain (21). PA K26E was also noted as a common variant arising within quail
977 experimentally inoculated with GFHK99 (22). While we see herein that the fitness effects of PA
978 26K are ameliorated in the context of multiple-infection, these fitness effects are nonetheless
979 likely to explain its low prevalence in nature. We speculate that the occurrence of PA 26K in
980 GFHK99 is the result of genetic drift, which can lead to fixation of deleterious mutations. While
981 PA 26K is not common in circulating IAVs, the phenotype it confers gives an opportunity to
982 identify viral and cellular processes that can underlie beneficial effects of multiple infection.

983 Cellular coinfection modulates the biology of diverse viruses. In VSV, inducing multiple
984 infections via virion aggregation can accelerate viral production such that it outpaces innate
985 antiviral responses (2). Similarly, in HIV-1, delivery of multiple viral genomes through cell-to-
986 cell transmission of infection leads to more rapid onset of viral gene expression (3, 4). For
987 rotavirus and norovirus, vesicle-bound packets of viral particles have enhanced infectivity
988 relative to free virions and are important vehicles for transmission (5, 6). Importantly for IAV,
989 beneficial effects of multiple infection can enable viral replication in novel host species (8).

990 This work reveals one mechanism by which multiple infection can be beneficial for IAV
991 replication; we predict that many distinct mechanisms will lead to a similar effect and that these
992 mechanisms will typically be active at the earliest stages of the viral life cycle, before incoming
993 vRNAs are replicated. Our data underline the potential for multiple infection to enable viral
994 replication under adverse conditions, such as in the presence of deleterious mutation or under

995 antiviral drug treatment. They furthermore reveal mechanistic insight into the high levels of
996 reassortment that are a major feature of IAV dynamics within hosts (23, 24).
997

998 **Author Contributions**

999 Concept and experimental planning were performed by JRS, CYL, and ACL. Data was collected
1000 and analyzed by JRS and CYL. Manuscript and figures were written, designed, and edited by
1001 JRS, CYL, and ACL. Research funding was acquired by ACL.
1002

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1007

1008 **Declaration of Interests**

1009 The authors declare no conflicts of interest.
1010

1011 **Methods**

1012 *Cells and cell culture*

1013 Madin-Darby canine kidney (MDCK) cells and 293T cells (ATCC, CRL-3216) were maintained
1014 in minimal essential medium supplemented with 10% fetal bovine serum and 100 ug/mL

1015 normocin (Invivogen). MDCK cells gifted by Peter Palese, Icahn School of Medicine at Mount
1016 Sinai were used for experiments. MDCK cells gifted by Daniel Perez, University of Georgia,
1017 were used for plaque assays. All cells were maintained at 37°C and 5% CO_2 in a humidified
1018 incubator and monitored monthly for mycoplasma contamination.

1019 *Viruses*

1020 Influenza A viruses used in these experiments were generated via reverse genetics (25, 26).
1021 Briefly, 293T cells transfected with ambisense plasmids encoding the eight viral gene segments
1022 were injected 16-24 h after transfection into the allantoic cavity of 10-11 day-old embryonated
1023 chicken eggs (Hyline International) and incubated for 32-36 h at 37°C. Allantoic fluid was
1024 collected and used as the virus stock for experiments. Infectious titers were determined by plaque
1025 assay in MDCK cells and by flow cytometry targeting virally encoded epitope tags. Levels of
1026 internally deleted defective interfering segments derived from PB2, PB1, PA, and NP segments
1027 were confirmed to be minimal for each virus stock, using previously described procedures (27).
1028 All viruses used contained PB2, PB1, HA, NP, NA, M, and NS segments derived from influenza
1029 A/mallard/Minnesota/199106/99 (H3N8) virus (MaMN99). MaMN99 PA viruses contained the
1030 PA gene segment from MaMN99. MaMN99: GFHK99 PA viruses contained the PA gene
1031 segment from influenza A/guinea fowl/Hong Kong/WF10/99 (H9N2) virus. The HA segment of
1032 each virus used was engineered to contain either a 6xHIS or an HA epitope tag and GGGS linker
1033 following the signal peptide. Homologous WT and VAR viruses were tagged with opposite
1034 epitopes to allow differentiation of infected cells via flow cytometry. GFHK99 Endo PA and
1035 MaMN99 viruses were tagged WT as HIS-tag and VAR as HA-tag. All other viruses were
1036 tagged WT as HA-tag and VAR as HIS-tag. Homologous VAR viruses included one
1037 synonymous mutation in each segment relative to the WT strain, as detailed previously (8).

1038 *Generation of modified PA plasmids*

1039 Plasmids encoding chimeric PA gene segments were created using the NEBuilder HiFi DNA
1040 Assembly Master Mix (New England Biosciences) according to the manufacturer's instructions
1041 using the primers in Table 1. PCR-amplified fragments encoding the endonuclease region
1042 (GFHK99 Endo), linker and arch region (GFHK99 Arch), or C-terminal region (GFHK99 C-
1043 term) of GFHK99 PA were combined with a fragment containing the rest of the MaMN99 PA
1044 segment in a pDP2002 plasmid, a gift from Daniel Perez (28). Mutations to the PA 26 codon
1045 were introduced using site-directed mutagenesis (QuikChange, Agilent) using primers listed in
1046 Table 2. Two nucleotide changes were introduced to avoid reversion. MaMN99 ΔPA-X and
1047 MaMN99:GFHK99 PA ΔPA-X were designed as described previously (17): site-directed
1048 mutagenesis of three bases at the X-ORF (t597c, t600c, and t627a) decrease the likelihood of
1049 frameshifting and add a TAG stop codon to truncate the C-terminal region of PA-X. Sequences
1050 of purified plasmid preparations were verified by Sanger sequencing (Genewiz). WT and VAR
1051 pairs of each recombinant virus were generated as described above. ΔPA-X segment loss-of-
1052 function was verified via host shutoff capacity: 293T cells were transfected with 40 ng of either
1053 pCAGGS-MaMN99-PA or pCAGGS-MaMN99-PA-ΔPA-X plasmids and 50 ng of pRL-TK
1054 plasmid (Promega). At 24 h post-transfection, the transfected cells were lysed and 20 µl of lysate
1055 was transferred to a 96-well plate. 100 µl of *Renilla* luciferase assay reagent (Promega) was
1056 added and then *Renilla* luciferase activity was measured on a BioTek Synergy H1 Hybrid
1057 Reader. *Renilla* luciferase activity was plotted relative to empty vector transfected cells.

1058 *Infection of cells for quantification of cellular coinfection and viral reassortment*

1059 Infections of cultured cells were performed as described previously (8, 29). Briefly, homologous
1060 WT and VAR viruses were mixed in equivalent amounts based on infectious titers as determined

1061 by flow cytometry, diluted serially in 1x PBS, and used to inoculate 80% confluent MDCK cells
1062 in 6 well dishes. Synchronized single-cycle infection conditions were used: to synchronize viral
1063 entry, virus was allowed to attach during a 45 min incubation at 4°C before addition of warm
1064 virus medium (1xMEM, 4.3% BSA, 100 IU penicillin/streptomycin) and incubation for 2 h at
1065 37°C. At the end of this 2 h incubation, residual inoculum was inactivated using a 5 min acid
1066 wash in PBS-HCl (pH=3). Cells were then placed in virus medium supplemented with 20 mM
1067 NH_4Cl and 50 mM HEPES and incubated at 37°C. This high pH buffer prevents endosomal
1068 acidification and therefore blocks any further viral entry, imposing single cycle conditions.
1069 Released virus and cells were collected at 16 hpi (with 0 hpi defined as the time of warming).
1070 Supernatant was stored at -80°C until use in plaque assays.

1071 Coinfections with baloxavir acid (MedChemExpress, CAS No. 1985605-59-1) were completed
1072 in the same manner as above, with 5nM BXA added to the virus medium both during the 2 h
1073 viral entry period and the 14 h viral replication period.

1074 *Quantification of infection and coinfection*

1075 Frequencies of infection and coinfection in cell monolayers co-inoculated with WT and VAR
1076 viruses were evaluated based on surface expression of HA- and HIS-tags. Samples were stained
1077 for 45 min on ice with Penta HIS Alexa Fluor 647 conjugated antibody (5 ug/ml; Qiagen) and
1078 Anti-HA-FITC Clone HA-7 (7 ug/ml; Sigma Aldrich). Cells were then washed and resuspended
1079 in PBS-2% FBS in cluster tubes for flow cytometry analysis on a BD-FACSymphony A3
1080 cytometer in the Emory University Flow Cytometry Core. Analysis was performed using FlowJo
1081 10.8.1 software. Non-linear regressions (least squares) and linear regressions of the data were
1082 performed in Prism Graphpad.

1083 *Quantification of reassortment*

1084 The frequency of reassortant viruses was determined as described previously (24). Plaque assays
1085 were performed in 10 cm-diameter dishes to isolate viral clones and agar plugs were collected
1086 with 1 mL serological pipettes into 160 µl PBS. vRNA was extracted using the Quick RNA 96
1087 extraction kit (Zymo) then reverse transcribed using Maxima reverse transcriptase
1088 (Thermofisher) per the manufacturer's instructions using the Universal F(A)+6 primer
1089 (gcgcgcagcaaaagcagg). cDNA was diluted 1:4 in nuclease-free water and combined with
1090 segment-specific primers (8) to differentiate WT and VAR segments by high-resolution melt
1091 analysis with Precision Melt Supermix (Bio-Rad) using a CFX384 Touch Real-time PCR
1092 detection system (Bio-Rad) and BioRad CFX Manager 3.1 software. Data were analyzed using
1093 Precision Melt Analysis 1.3 software (Bio-Rad) to assign a genotype based on the combination
1094 of WT and VAR segments in each isolate. Percent reassortment was calculated as the number of
1095 viral isolates with any reassortant genotype divided by the number of isolates screened,
1096 multiplied by 100. Results were plotted as a function of percent HA+ cells as determined by flow
1097 cytometry. Semi-log curves were fitted to the data in Graphpad.

1098 *Analysis of the shutoff of cellular gene expression*

1099 250, 50, 10, 2, or 0.4 ng of MaMN99 or MaMN99 PA E26K plasmids were ectopically
1100 transfected to 293T cells with 50 ng of pRL-TK plasmid using X-tremeGENE 9 (Roche). At 24
1101 h, the transfected cells were lysed and 20 µl of lysate was transferred to a 96-well plate. 100 µl of
1102 *Renilla* luciferase assay reagent (Promega) was added and then *Renilla* luciferase activity was
1103 measured on a Synergy H1 Hybrid Reader (BioTek).

1104 *Strand-specific quantification of vRNA and mRNA over time*

1105 MDCK cells (1 $\times 10^5$ cells per well) were seeded onto 24-well plate and incubated at 37°C for 24
1106 h. The cells were washed three times with PBS, then chilled viruses were inoculated under single
1107 cycle infection conditions, low MOI of 0.5 RNA copies/cell and high MOI of 1000 RNA
1108 copies/cell. After 1h of absorption, the cells were washed three times with PBS. The cells were
1109 collected at 0, 2, 8, and 12 h post-infection, and the RNA was extracted using RNeasy Mini kit
1110 (Qiagen). To reverse-transcribe specific RNA species, two different primers targeting vRNA or
1111 mRNA of NS segment were used (vRNA primer: ggccgtcatggtggcgaat; mRNA primer:
1112 ccagatcgttcgagtcgt) (8). The quantitative PCR was conducted with Ssofast EvaGreen Supermix
1113 (Bio-rad) and specific primer set targeting vRNA or mRNA of NS using CFX384 Touch Real-
1114 time PCR (Bio-rad) (Table 3) (30).

1115 *Western blotting*

1116 Western blotting was performed using the Thermo scientific miniblot module. SDS-PAGE of
1117 reduced samples was run on Bolt Bis-Tris 4-12% premade gels in MOPS. Blotting was
1118 performed onto 0.2nm nitrocellulose membranes at 15V for 30 minutes. Blots were blocked with
1119 5% milk in PBS-0.05% Tween 20 for 1h at room temperature, then incubated overnight at 4°C
1120 with primary antibodies at 1:2,000: rabbit anti-PA polyclonal (Genetex), mouse anti-β-actin
1121 (Sigma, clone AC-74), and mouse anti-influenza NP (Kerafast, clone HT103). Secondary
1122 antibodies (1:3,000) goat anti-Rabbit IgG-HRP (Sigma) and goat anti-mouse IgG-HRP
1123 (Promega) were incubated with the blots for 1h at room temperature and the blots were
1124 developed using Bio-Rad Clarity Western ECL Substrate. Images were taken on the Bio-Rad
1125 ChemiDoc MP imaging system.

1126 *Protein modeling*

1127 Models of PA were created using the Phyre2 protein fold recognition server

1128 (http://www.sbg.bio.ic.ac.uk/phyre2/) to predict structures based on the published sequence of

1129 GFHK99 PA (Genbank accession # MN267497.1) (31). Visualization was performed with UCSF

1130 Chimera (https://www.rbvi.ucsf.edu/chimera/), developed by the Resource for Biocomputing,

1131 Visualization, and Informatics at the University of California, San Francisco, with support from

1132 NIH P41-GM103311 (32).

1133 *Quantification and statistical analysis*

1134 Analysis of these data was performed using the GraphPad Prism statistical software. Replicate

1135 sizes are indicated on figure legends where applicable. Linear and non-linear (least-fit)

1136 regressions were performed on data collected via flow cytometry. Semi-log lines were fitted to

1137 reassortment data. PA-X host shutoff capacity was analyzed using unpaired Student's t-tests and

1138 reported +/- SD for Fig. 2A. Data in Fig. 5 were analyzed via 2-way ANOVA. Alpha = 0.05.

1139

1140 **Tables**

1141

1142 **Table 1:** Primers used for chimeric PA segment Gibson Assembly

Oligonucleotide	Sequence
Endo GFHK99 F	ctgatccaaaatggaagactttgtgcgac
Endo GFHK99 R	attcgccccgggactgacgaaaggaatc
Endo pDP-MaMN99 F	tcgtcagtcccggggcgaatcaataattg
Endo pDP-MaMN99 R	agtcttccattttggatcagtacctgctttc
Arch GFHK99 F	ggatggattcgaaccgaacggctgcattg
Arch GFHK99 R	tctgaatccagcttgctagcgatctaggc
Arch pDP-MaMN99 F	gctagcaagctggattcagagtgaattc
Arch pDP-MaMN99 R	cgttcggttcgaatccatccacataggc
C-term GFHK99 F	gctagcaagctggatccagagtgagttc
C-term GFHK99 R	gccacaactattttagtgcatgtgtgag
C-term pDP-MaMN99 F	tgcactaaaatagttgtggcaatgctac
C-term pDP-MaMN99 R	tctggatccagcttgctagcgatctagg

1143

1144 **Table 2:** Primers for ΔPA-X site-directed mutagenesis

Oligonucleotide	Sequence
MaMN99PAX t627a F	gatttcaaatctttcttctattgtctcttcgcctctttcgga
MaMN99PAX t627a R	tccgaaagaggcgaagagacaatagaagaaagatttgaaatc
MaMN99PAX t597c_t600c F	ctctttcggactggcggaaggaatcccatagacccc
MaMN99PAX t597c_t600c R	ggggtctatgggattccttccgccagtccgaaagag
GFHK99PAX t627a F	atttcaaatctttcttctattgtctcttcgcctctctcgg
GFHK99PAX t627a R	ccgagagaggcgaagagacaatagaagaaagatttgaaat
GFHK99PAX t597c_t600c F	ctctctcggactggcggaaggaatcccatagacccc
GFHK99PAX t597c_t600c R	ggggtctatgggattccttccgccagtccgagagag

1145

1146 **Table 3:** Primers used for strand-specific qPCR of MaMN99

Oligonucleotide	Sequence
MaMN99 vRNA NS 552F	ggccgtcatggtggcgaat aatgcaattggaatcctcat
MaMN99 mRNA NS 13R	ccagatcgttcgagtcgttttttttttttttttatcattaaataag
MaMN99 NS 795F	cttgcaggcattgcaac
MaMN99 NS 643R	cggactccccaagcgaatctc

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1150 References

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Chapter 3

Conclusions

PA 26K

Influenza A viruses infect many species of birds and mammals, including humans. The propensity of IAVs for genetic diversification poses a risk of introducing a novel strain into a naïve population, however many barriers limit the successful transmission and replication of viruses in a new host. This work examined the observation that some IAVs require multiple infection of a single cell to productively replicate. GFHK99, an H9N2 virus of the G1 lineage of avian viruses, was found in previous work to display a high reliance on multiple infection that is determined by the PA segment. The research herein narrowed the viral determinant to a specific residue, PA 26K, located in the endonuclease region of the PA protein. PA 26K is associated with decreased polymerase and endonuclease function. PA 26E, which is seen in other H9N2 viruses, improves the function of both and removes the stringent requirement for multiple infection. These data have revealed that a single amino acid in the PA segment is the largest contributor to attenuation of GFHK99 in mammalian cells and that multiple infection allows it to surmount this barrier.

Evaluation of ferrets as a model organism for H9N2 showed that GFHK99 could replicate in and pass to a direct contact animal but did not spread to other ferrets by aerosols (1). It is possible that too few viruses to allow multiple infection were able to be passed over distances. In quail transmission studies using GFHK99, PA K26E was detected to become a fixed polymorphism in contact animals at high frequencies within a single host infection cycle indicating that 26E confers a fitness advantage in quail, however PA 26K was still seen to transmit between quail (2, 3). PA 26K occurs rarely in databases of sequenced influenza A viruses and 99.87% of H9 viruses instead have a glutamic acid at that position (4). The uniqueness of PA 26K among IAVs means that it is

1264 not a predominant driver of multiple infection reliance and there are other viral determinants
1265 affecting other strains.
1266 PA 26K is certainly not the only determinant of reliance on multiple infection in IAVs. In
1267 previous work from this lab, influenza A/quail/Hong Kong/A28945/88 (H9N2) virus (QaHK88)
1268 was identified as also relying on multiple infection (5). In coinfection experiments in MDCK cells,
1269 increasing concentrations of QaHK88 VAR virus led to a marked increase in WT vRNA copy
1270 number, even higher than that of GFHK99. This was interpreted to show that QaHK88 relied
1271 extensively on multiple genomes in order to replicate. When a MaMN99: QaHK88 PA virus was
1272 used in quantification of infection and coinfection experiments, however, it was seen that the
1273 frequency of coinfected cells did not relate linearly to the frequency of infected cells as would be
1274 expected for high reliance. This indicates that multiple infection assists the replication of QaHK88,
1275 as vRNA production is enhanced, but this phenotype is not linked to PA as GFHK99's phenotype
1276 is. In this context, further research into the impact of multiple genomes on IAV infection and
1277 replication is needed to determine what factors are involved in strains that do not include PA 26K.
1278
1279 *Public health implications*
1280 From a virus surveillance standpoint, the finding that multiple genome delivery can
1281 facilitate productive infections in otherwise non-susceptible hosts adds to the complexity of
1282 determining the risks of novel strains. Since 1988, H9N2 viruses have been found in increasing
1283 frequencies in poultry in many countries and, while considered LPAI, can impact the quality of
1284 animal life and products (6, 7). Sporadic zoonoses of H9N2 to humans who live or work closely
1285 with poultry have occurred since 1998 and serologic data show that exposure to H9N2 viruses is
1286 likely more common than recorded (8-10). Interestingly, H9N2 viruses isolated since 2000 from

1287 both birds and humans express HA with 226L, which lends preference to the human-prevalent
1288 sialic acid α2,6 linkage (11). Previous isolates instead expressed HA 226Q which prefers α2,3. A
1289 number of other mammalian-adaptive polymorphisms in HA and other IAV proteins have also
1290 been described in H9N2 viruses that have put it on the short list of potential pandemic viruses (12-
1291 14).

1292 H9N2 viruses have demonstrated extensive reassortment in the past decades, which raises
1293 the possibility of a future reassortment event that can allow more efficient spread between humans
1294 (15-17). Continual circulation in poultry and verified transmission to pigs, the stereotypical
1295 "mixing vessel," creates a situation conducive to coinfection. Already, it has been determined that
1296 H9N2 provided internal genes to a strain of H5N1 IAV responsible for a human outbreak in Hong
1297 Kong in 1997 after both subtypes co-circulated amongst animal markets (18). H9N2 has also been
1298 found to have contributed internal genes to HPAI H7N9 and H10N8 viruses, both of which have
1299 sporadically infected humans (12, 19-21). H9N2 in pigs has the potential to reassort with H3N2
1300 seasonal circulating viruses that are repeatedly introduced into pigs from humans (22, 23).

1301 This dissertation's finding that multiple infections of GFHK99 result in high frequencies
1302 of reassortant progeny may have implications for the reassortment and pandemic potential of avian
1303 viruses. A virus strain that is ill-adapted to spreading to a new host species may complete a
1304 productive lifecycle and amplify in this host if the infectious dose is high enough to facilitate
1305 multiple infection. It then has a chance to accumulate mutations, some of which may improve
1306 fitness in the new host. If a new host has also been infected by a heterologous virus, these viruses
1307 could reassort. As H9N2 viruses are predisposed to infection of mammals and are circulating in
1308 areas with other viruses, multiple-infection assisted reassortment is feasible.

1309

1310 *The past and future of collective interactions*

1311 The observation that multiple viruses can infect a single cell and alter infection outcome
1312 has been noted in scientific journals since the early days of virological research. By the end of the
1313 1940s, bacteriophage researchers Luria and Dulbecco (of growth media fame) described
1314 multiplicity reactivation, where a partially inactivated phage could still reproduce if another phage
1315 were introduced to the cell to "complete the set" of genetic units (24). While the validity of their
1316 conclusion appears to have been debated at the time, other virologists studying the phenomenon
1317 of viral interference (soon to be named interferon) found similar results (25). Coinfection of
1318 polioviruses was postulated by the mid-1950s, one researcher claiming that it "seems likely that
1319 [multiplicity reactivation] MR in bacteriophage has a genetic mechanism (although conclusive
1320 evidence on this point is lacking), poliovirus MR constitutes the first suggestion of genetic
1321 interactions among poliovirus" (26).

1322 Multiplicity reactivation was described for IAVs by the 1950s, as well. Though subjected
1323 to partial inactivation through UV or heat exposure, influenza viruses could still replicate in eggs
1324 in an MOI-dependent manner, leading to the researchers to speculate that multiple infection was
1325 occurring (27). Infection kinetic increases were also observed when using higher concentrations
1326 of influenza virus while investigating multiplicity reactivation, which has since been validated in
1327 other viruses (28, 29).

1328 A large variety of viral interactions besides reactivation, both beneficial and detrimental to
1329 the viral population, are now known to affect infection and viral output (30, 31). As molecular
1330 methods have increased in resolution and capability, it has become possible to examine the
1331 dynamics of infection on a finer scale. This improved insight, perhaps paradoxically, brings the
1332 capacity to apply broader biological frameworks to virology. One of these is sociobiology.

1333 Popularized by the late E.O. Wilson, this area of biology examines the relationship between social
1334 behavior and evolution (32). While viruses might not be social in the way that a macro-organism
1335 is, they are still packets of genetic information that interact, and the type of interaction determines
1336 how well that genetic information is passed to a new generation.

1337 Density dependence, the mechanism postulated above for GFHK99's coinfection
1338 phenotype, is a well-explored concept in the biology of non-human animals wherein changes in
1339 population density impact population growth. Positive density dependence, more specifically, is a
1340 situation where the denser the population, the higher the growth rate (33). This is also known as
1341 the Allee effect. In a small population of turtles in a patch of wetland, say, an individual male
1342 might have trouble finding a female with which to mate, maybe because few females are
1343 reproductively mature or because he rarely encounters another turtle. As more turtles enter the
1344 wetland or reach reproductive age, the probability of an individual turtle finding a mate improves.
1345 Similarly, as more viruses are added to a cell, the effect of stochastic factors that might lead to
1346 failed infection are felt less severely and more resources are available.

1347 Viewing IAV multiple infection reliance through the lens of "sociovirology" requires
1348 consideration of the evolution component: how has multiple infection been shaped by selective
1349 pressure? This has been addressed briefly in viruses, as the Allee effect was described in a VSV
1350 model where virus aggregation improves fitness over single viruses (34). Several viruses entering
1351 a cell at once complete the first round of replication more quickly than equal numbers of single
1352 viruses (29, 34). Rapid replication as a result of multiple infection may, therefore, allow viruses to
1353 circumvent stochastic hurdles and begin an infectious cycle more quickly to establish a foothold
1354 before immune responses have a chance to limit their spread. This would select for viruses with a
1355 propensity for multiple infection despite, as with GFHK99, a replication defect.

1356 There are likely many drivers of multiple infection reliance in IAVs and other RNA viruses.
1357 For GFHK99, a specific endonuclease residue in PA attenuates the virus in mammalian cells and
1358 this is compensated for with the addition of genomes. Whether aggregation or other methods of
1359 concentrating viral particles are employed to facilitate multiple infection is still to be seen. Another
1360 interesting avenue to pursue is whether early events in the viral lifecycle could be potential
1361 therapeutic targets to prevent rapid establishment of infection. Cognizance of multiple infection is
1362 vital to understanding viral adaptation and GFHK99 presents a good model with which to study
1363 this dynamic.
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