1 **Influenza Virus-Host Interactomes as a Basis for Antiviral Drug**
2 **Development**

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4 Tokiko Watanabe¹, Yoshihiro Kawaoka^{1,2,3}

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6 *¹Division of Virology, Department of Microbiology and Immunology, Institute of*
7 *Medical Science, University of Tokyo, Tokyo 108-8639, Japan*

8 *²Department of Pathobiological Sciences, School of Veterinary Medicine, University*
9 *of Wisconsin-Madison, 575 Science Drive, Madison, WI 53711, USA*

10 *³Department of Special Pathogens, International Research Center for Infectious*
11 *Diseases, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-*
12 *8639, Japan*

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16 *Correspondence should be addressed to Yoshihiro Kawaoka, Department of
17 Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-
18 Madison, 575 Science Drive, Madison, WI 53711, USA; Phone: (608) 265-4925, Fax:
19 (608) 265-5622, E-Mail: kawaokay@svm.vetmed.wisc.edu

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Abstract

Currently, antiviral drugs that target specific viral protein functions are available for the treatment of influenza; however, concern regarding the emergence of drug-resistant viruses is warranted, as is the urgent need for new antiviral targets, including non-viral targets, such as host cellular factors. Viruses rely on host cellular functions to replicate, and therefore a thorough understanding of the roles of virus-host interactions during influenza virus replication is essential to develop novel anti-influenza drugs that target the host factors involved in virus replication. Here, we review recent studies that used several approaches to identify host factors involved in influenza virus replication. These studies have permitted the construction of an interactome map of virus-host interactions in the influenza virus life cycle, clarifying the entire life cycle of this virus and accelerating the development of new antiviral drugs with a low propensity for the development of resistance.

Introduction

Influenza viruses cause annual epidemics and recurring pandemics, such as the Spanish influenza in 1918–1919, the Asian influenza in 1957, the Hong Kong influenza in 1968, and the swine-origin H1N1 2009 pandemic influenza in 2009; these outbreaks have a huge impact on public health and the global economy [1]. In addition, recent sporadic human infections with H5N1 and H7N9 avian influenza viruses have raised concerns regarding the pandemic potential of these viruses [2-5]. Currently, two classes of FDA-approved compounds are available for prophylaxis and treatment of influenza virus infection: M2 ion channel inhibitors (amantadine, rimantadine) and neuraminidase (NA) inhibitors (oseltamivir, zanamivir) [6,7]. In addition, several new antiviral compounds have been approved for human use in some countries, including two NA inhibitors (peramivir and laninamivir) [8-11], and a viral polymerase inhibitor [favipiravir (T-705)] [12-14]. However, a major problem concerning anti-influenza drugs that target viral proteins is the frequent emergence of drug-resistant viruses. Indeed, most human H3N2 and H1N1, including pandemic 2009 H1N1, influenza viruses are now resistant to amantadine/rimantadine [15,16], and oseltamivir-resistant H1N1 viruses have emerged in many countries frequently [17]. Therefore, concern regarding the emergence of drug-resistant viruses is clearly warranted, as is the urgent need for new antiviral targets, including non-viral targets, to treat influenza virus infection while minimizing the emergence of drug resistance.

Host factors have been in the spotlight recently as potential antiviral targets to overcome the problems described above. Emergence of resistance could be less frequent when host factors are targeted compared with directly targeting viral proteins. Recently, several drugs targeting host factors identified to be important for

63 virus replication have been developed. For example, the antiretroviral drug maraviroc,
64 which was approved by the FDA in 2007, targets C-C chemokine receptor type 5
65 (CCR5), a co-receptor for human immunodeficiency virus type 1 (HIV-1), preventing
66 HIV-1 entry [18]. Another example is alisporivir, a cyclophilin inhibitor, which was
67 under clinical evaluation for the treatment of chronic hepatitis C [19,20]; in 2012,
68 however, the FDA halted the clinical trials due to the possible side effect of
69 pancreatitis. Alisporivir inhibits the function of cyclophilin A, which has an essential
70 role in hepatitis C virus (HCV) replication and virus production [21]. In the case of
71 influenza, the sialidase DAS181 (Fludase), which is currently in clinical trials, targets
72 influenza virus receptors, sialic acids, and prevents viral attachment via removal of
73 the sialic acids from the epithelial cells of the respiratory tract (Figure 1) [22-27].
74 Several other compounds, including protease inhibitors, block cellular proteases,
75 which mediate the HA cleavage that is required for membrane fusion between the
76 viral envelope and the endosomes [28-34], the MEK inhibitor U0126, NF- κ B
77 inhibitors such as acetylsalicylic acid (known as aspirin), and agonists of sphingosine-
78 1-phosphate receptors AAL-R, all of which target host cellular functions involved in
79 influenza virus replication, have been studied to explore their potential as new
80 antiviral drugs against influenza (Figure 1) [26,35-41]. In addition, combination
81 therapy with a protease inhibitor and conventional anti-influenza drugs has been
82 recently tested *in vitro* [42].

83 The advent of the possibility to target host factors involved in virus replication
84 places us at the threshold of a new era of antiviral drug development. However, to
85 explore targets for antiviral therapy, a systematic understanding of the mechanisms of
86 influenza virus replication is essential. Recent efforts to identify host factors involved
87 in influenza virus replication have provided the missing pieces of the virus-host

88 interaction map of the influenza virus life cycle, helping us to move toward the
89 development of host-targeting antiviral drugs. Here, we review the recent progress in
90 the identification of host factors involved in influenza virus replication and the latest
91 efforts to develop antiviral drugs that target host factors.

92

93 **Strategies to identify host factors involved in influenza virus replication**

94 Recent technological advances have made it possible for researchers to take various
95 approaches to identifying host factors involved in the life cycles of several viruses,
96 including HIV-1, HCV, Dengue virus, and West Nile virus, as well as influenza virus
97 [43-54]. For influenza virus, several strategies have been applied to the search for host
98 factors involved in viral replication, as described below (Figure 2).

99

100 ***Genome-wide screens***

101 The yeast single-gene deletion library consists of almost 5,000 single-gene deletion
102 strains and covers about 80% of the yeast genome. This library has been used
103 extensively in the yeast community for genomic studies. Naito et al. [55] used it to
104 identify host genes involved in influenza virus replication. They used *Saccharomyces*
105 *cerevisiae* to establish a system that supported influenza viral genome replication
106 and transcription and then screened a sub-library that lacked 354 genes encoding
107 putative nucleic acid-binding and related functional proteins. Several host factors that
108 affect influenza viral RNA synthesis, including Tat stimulatory factor 1 (Tat-SF1),
109 were identified in this study[55].

110 Genome-wide RNAi-based screens also represent an excellent approach to
111 searches for host factors involved in virus replication. In the absence of RNAi-based
112 screening systems in mammalian cells, Hao et al. [48] used *Drosophila* RNAi

113 technology to screen for host factors involved in influenza virus replication. They
114 tested an RNAi library against more than 13,000 genes (approximately 90% of the
115 *Drosophila* genome) and identified 110 *Drosophila* genes that, when depleted,
116 affected influenza virus replication in *Drosophila* cells. Of those 110 candidates,
117 further analysis validated roles for three host proteins—ATP6V0D1 (an ATPase),
118 COX6A1 (a cytochrome C oxidase subunit), and NXF1 (a nuclear RNA export
119 factor)—in influenza virus replication in mammalian cells.

120 When RNAi-based screening systems in mammalian cells were subsequently
121 established, the roles of mammalian host proteins in influenza virus replication could
122 be comprehensively analyzed. Three key genome-wide RNAi-based screen studies
123 were published in 2009 and 2010 [43,46,47]. Brass et al. [46] identified 133 host
124 factors potentially involved in the influenza life cycle steps of virus entry, uncoating,
125 vRNP nuclear import, genome transcription, and viral protein translation by
126 examining the effects of siRNAs targeting over 17,000 human genes on influenza
127 virus replication in human osteosarcoma. König et al. [43] performed a similar study
128 using the human lung cell line A549 and identified 295 host factors that may also be
129 involved in these early to middle steps of the influenza life cycle. In the third key
130 genome-wide RNAi-based screen study, Karlas et al. [47] looked at the entire
131 influenza virus life cycle. By measuring virus titers in the culture supernatant of
132 siRNA-treated, virus-infected cells they identified 287 host factors important for
133 influenza virus replication [47].

134 Sui et al. [56] applied ‘Random Homozygous Gene Perturbation’ (RHGP) to
135 identify host targets that are required for influenza virus infection. RHGP uses a
136 lentiviral-based genetic element that integrates into the genome leading to abrogation
137 or enhancement of gene expression and an altered phenotype. The authors generated

138 an RHGP library in mammalian cells, which they then infected with influenza virus.
139 When they isolated the cell clones that survived and sequenced their genomes, they
140 were able to identify 110 host genes that were associated with host cell resistance to
141 influenza virus infection.

142

143 ***Proteomics approaches***

144 Yeast two-hybrid analyses and proteomics approaches have been successfully used to
145 identify host molecules that interact with the influenza viral proteins (reviewed in
146 [57]), and identified several host proteins involved in viral genome replication and
147 transcription [58-65], as have NS1 protein-interacting host factors involved in the
148 immune response to infection [66]. This system also identified 87 human proteins as
149 interaction partners of ten influenza viral proteins and 109 human proteins as
150 interaction partners of three viral polymerase subunits [49,67], making it possible to
151 built interactome maps consisting of influenza viral-host protein interactions.

152 By using a mass spectrometry approach, Mayer et al. [68] identified 41 host
153 proteins that interact with the influenza viral ribonucleoprotein complex. Of these 41
154 proteins, they demonstrated a potential role for nucleoplasmin in viral RNA synthesis.
155 Shaw et al. [69] and Hutchinson et al. [70] also used a mass spectrometry approach to
156 identify host cellular proteins that are incorporated into progeny virion particles. They
157 found that influenza virions contain abundant host proteins that make a substantial
158 contribution to the influenza virion architecture. Shaw et al. [69] identified 36 host
159 proteins incorporated in virions, including cytoskeletal proteins, annexins, glycolytic
160 enzymes, and tetraspanin. Hutchinson et al. [70] found that the host proteins that are
161 part of influenza virions produced in virus-infected mammalian cells largely

162 overlapped with those produced in virus-infected chicken eggs, suggesting that these
163 common host proteins are important in the formation of influenza virion particles.

164

165 *Combining proteomics and RNAi-based screen approaches to construct the*
166 *interactome map of viral-host protein interactions in influenza virus replication*

167 Shapira et al. [49] used a combination of proteomics and functional genomics to
168 construct an interactome map of the viral-host protein interactions that occur during
169 influenza virus replication. They used a yeast two-hybrid screen to identify host
170 proteins that interact with viral proteins and then built a physical map of these
171 interactions. They also used transcriptional profiling to determine whether the host
172 cell genes were differentially expressed in primary human bronchial epithelial cells
173 upon influenza virus infection (or exposure to influenza viral RNA). By using this
174 approach, they identified 1,745 host factors with potential involvement in influenza
175 virus replication. When they tested siRNAs targeted to these 1,745 human genes, they
176 found 616 host factors with involvement in influenza virus replication.

177 Watanabe et al. [71] used mass spectrometry and RNAi-based screening
178 approaches to elucidate the physical and functional host-viral interactions during
179 influenza virus replication. They first identified 1,292 host proteins that co-
180 immunoprecipitated with influenza viral proteins that were transiently expressed in
181 human cells. Then, they validated the roles of 324 of these host factors in influenza
182 virus replication by testing the effects of siRNAs targeted to the 1,292 influenza viral
183 host interaction partners on influenza virus production. Further extensive analyses of
184 the 91 top-ranked host factors allowed them to define the steps of the viral life cycle
185 that were affected and to generate an interactome map of the virus-host protein
186 interactions that are involved in the influenza virus replication cycle.

187

188 **Exploring antiviral drugs designed to target host factors involved in influenza**
189 **virus replication**

190 The studies described above have produced a vast amount of data on the host factors
191 that are involved in influenza virus replication. The next step is to select host factors
192 that may be suitable targets for antiviral drugs. Small compounds that inhibit the
193 cellular functions of particular host proteins have been used to validate the
194 requirement of a specific host protein for virus replication (Figure 1). Konig et al. [43]
195 tested the compound KN93, which is a selective inhibitor of CAMK2B
196 (calcium/calmodulin-dependent kinase II β) that was shown to be involved in the
197 regulation of viral RNA transcription in their genome-wide screen, and demonstrated
198 that the compound reduced virus titers in influenza virus-infected cells. Karlas et al.
199 [47] showed that treatment with the compound TG003, a small molecule inhibitor of
200 CDC-like kinase 1 (CLK1) identified as a host candidate involved in influenza virus
201 replication in their screen, led to reduced virus production in human cells probably by
202 affecting the splicing of viral RNAs. Brass et al. [46] found that the interferon-
203 inducible transmembrane proteins IFITM1, 2, and 3 hampered the early steps of
204 influenza virus replication *in vitro*, and also suggested that expression of IFITM3
205 defends host against influenza virus infection *in vivo* [72]. Watanabe et al. [71] tested
206 11 compounds that inhibit the cellular functions of several host factors identified in
207 their screen. Of these compounds they found that ruxolitinib and gogicide A (which
208 are inhibitors of JAK1 and the Golgi-specific brefeldin A-resistant guanine nucleotide
209 exchange factor GBF1, respectively) markedly decreased virus titers in human cells
210 infected with influenza virus without affecting cell viability.

211 In addition to approaches targeting host cellular proteins, Morita et al. [73]
212 assessed the possible involvement of lipid products in influenza virus replication. By
213 conducting mediator lipidomics and a bioactive lipid screen, they discovered that the
214 DHA-derived protectin D1 isomer (10S, 17S-dihydroxydocosahexaenoic acid)
215 markedly hampers influenza virus replication *in vitro* by inhibiting the nuclear export
216 of viral mRNA. More importantly, they demonstrated that intravenous administration
217 of protectin D1 protected mice from influenza virus infection, suggesting that
218 protectin D1 has potential as a new therapeutic tool in the treatment and prevention of
219 influenza.

220 Besides the experimental studies described above, bioinformatics analysis
221 could be an attractive approach to identifying and prioritizing host factors with
222 potential as therapeutic targets (Figure 2). Chassey et al. [74] conducted a meta-
223 analysis using the datasets from the six independent genome-wide screens described
224 above [43,46-49,56] and made a list of 925 host factors important for influenza virus
225 replication (note that this list differs from lists obtained by others [75-77] due to the
226 use of a different data evaluation strategy). By retrieving information from the
227 DrugBank database (<http://www.drugbank.ca/>), Chassey and colleagues searched for
228 molecules that interacted with these 925 host factors [74]. They found 298 molecules
229 that potentially interact with 100 host factors. Importantly, of the 298 molecules, 204
230 are already approved by FDA, and therefore, could be highly prioritized candidates
231 for influenza therapy [74].

232 Matsuoka et al. [78] attempted to apply another bioinformatics approach to
233 predict and prioritize host factors that could be therapeutic targets by using a
234 comprehensive map of the influenza virus life cycle called 'FluMap' (accessible at
235 <http://www.influenza-x.org/flumap/>) (Figure 2). FluMap can be used to visualize the

entire influenza virus life cycle and was constructed on the basis of information obtained from the literature and publicly available pathway databases. The authors applied a computational network analysis, called a controllability analysis [79,80], to FluMap to identify host molecules that dysregulated the virus replication cycle when their functions were disrupted, resulting in inhibition of virus replication. By using the controllability analysis, Matsuoka and colleagues identified 112 critical host molecules [78]. Further *in silico* analysis led them to prioritize several important molecules, such as the nuclear pore complex, Akt, PKC, and the Ran/GTPase complex, which are essential and highly connected within the virus-host network [78]. Accordingly, these molecules can be prioritized as potential new therapeutic targets.

Concluding remarks

Given the high rates of emergence of influenza virus strains that are resistant to the currently available anti-influenza drugs that target viral protein functions, host cellular functions involved in influenza virus replication are attractive targets for antiviral therapy. The host cellular systems are formed by highly complicated and sophisticated networks that consist of interactions between numerous cellular components; viruses hijack these host cellular system, leading to the re-construction of different networks to meet their need to replicate. Recent progress in our understanding of the virus-host interactions during influenza virus replication and of the human interactome network can accelerate the construction of a precise map of the virus-host interactome network during influenza virus replication, leading to a systematic understanding of the mechanisms involved in the influenza virus life cycle. Combining detailed functional approaches (e.g., *in vitro* and *in vivo* analyses) and extensive *in silico* approaches with such a comprehensive map of influenza virus

261 replication could help to identify and prioritize novel host factors with potential as
262 therapeutic targets.

263

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266 highlighted as:

267 • of special interest

268 •• of outstanding interest

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601

602 **Figure legends**

603

604 Figure 1.

605 A schematic diagram of compounds that inhibit influenza virus replication and their
606 known or putative points of action. There are two types of compound: one targets
607 viral protein functions and the other targets host cellular functions. Examples of the
608 former include M2 ion channel inhibitors (e.g., amantadine, rimantadine), NA
609 inhibitors (e.g., oseltamivir, zanamivir, peramivir, and laninamivir) and viral
610 polymerase inhibitors (e.g., favipiravir, **rivavirin**, and viramidine). Examples of the
611 latter include sialidase (e.g., DAS181) [22-27], dynamin inhibitors (e.g., dynarose)
612 [81], micropinocytosis inhibitors (e.g., EIPA) [81], MEK (MAPK/ERK kinase)
613 inhibitor (e.g., U0126) [35,36], V-type ATPase (vacuolar-type H⁺-ATPase)
614 inhibitors (e.g., bafilomycin A) [82], protease inhibitors (e.g., aprotinin [28], **which**
615 **inhibits proteases that cleave cell surface HA proteins that have a single arginine at**
616 **their cleavage site, and furin-like protease inhibitors, including peptidemimetics**
617 **derived from decanoylated basic tetrapeptides, such as decRVKR**
618 **chloromethylketone, which inhibit the HA cleavage of highly pathogenic H5 and H7**
619 **viruses [32-34] in the trans-Golgi, resulting in the inhibition of membrane fusion**
620 **between the viral envelope and the endosomes),** Hsp90 inhibitors (e.g., geldamycin)
621 [83], NF-κB inhibitors (e.g., aspirin) [37], PI3K (phosphatidylinositol 3-kinase)
622 inhibitors (e.g., LY294002) [84], ACC (acetyl-CoA carboxylase-α) inhibitors (e.g.,
623 TOFA) [85], and proteasome inhibitors (e.g., bortezomib) [86]. Compounds targeting
624 host factors that have been shown to be involved in influenza virus replication in
625 recent RNAi-based screens are also shown (i.e., KN93, TG003, ruxolitinib, and
626 golgicide A) [43,47,71].

627

628 Figure 2.

629 Overview of the strategies used to explore antiviral drugs targeting host factors
630 involved in influenza virus replication. Various approaches, such as genome-wide
631 screens, proteomics, transcriptomics, and lipidomics have been used to identify host
632 factors involved in the influenza virus life cycle. More detailed experimental studies
633 and bioinformatics analyses will help identify and prioritize host factors with potential
634 as therapeutic targets.

635

Fig. 1. Watanabe and Kawaoka

Fig. 2. Watanabe and Kawaoka