Different subtypes of influenza A viruses target different human proteins and pathways leading to different pathogenic phenotypes

Yujie Wang[1,2*], Ting Song[1*], Kaiwu Li[1], Yuan Jin[1], Junjie Yue[1§], Hongguang Ren[1§], Long Liang[1§].

[1]State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Biotechnology, Beijing 100071, China

[2]Institute of Physical Science and Information Technology, Anhui University, Hefei, Anhui 230601, China

[*]These authors contributed equally to this work.

[§]Correspondence: bioren@163.com (H.R.); ll@bmi.ac.cn (L.L.); yue_junjie@126.com (J.Y.)

Abstract

Different subtypes of Influenza A viruses cause different pathogenic phenotypes after infecting human bodies. Direct binary interactions between viral proteins and human proteins provide an important background for influenza viruses to cause complex pathologies of hosts. Here, we demonstrated the different impacts on the TNF-α-induced NF-κB activation of H1N1 and H5N1 virus proteins. By further examining the virus-host protein-protein interactions (PPI), we found that the same segment protein of the H1N1 and H5N1 viruses target on different host proteins. We then performed a yeast two-hybrid analysis of a highly pathogenic avian H5N1 influenza virus and human proteins. Influenza-host protein-protein interaction networks of three strains of

influenza A viruses (including two other reported influenza-host PPI networks) were systematically compared and mapped on the network level and the pathway level. The results show subtype-specific characters of the influenza-host protein interactome, which may response for the specific pathogenic mechanisms of different subtypes of influenza viruses.

Importance

Influenza A virus (IAV) can cause contagious respiratory illness, namely influenza (flu). The symptoms of infections from different subtypes of IAVs vary from mild to severe illness. The mechanism of these different pathogenic phenotypes remains poorly understood. Our results show that the same NA and NP segments from H1N1 and H5N1 virus cause different impacts on the TNF-α-induced NF-κB pathway. Furthermore, we generated a yeast two-hybrid protein-protein interaction (PPI) network between H5N1 and human proteins. By systematically comparing the influenza-host PPI networks of three strains of IAVs, we show that different subtypes of IAVs target different human proteins and pathways, which may have led to different pathogenic phenotypes.

Introduction

Influenza A virus (IAV) belongs to the Orthomyxoviridae family. Its genome consists of eight segmented, negative-strand RNA [1-3]. IAV are further typed into many different

38 subtypes based on the antigenicity of hemagglutinin (HA) and the neuraminidase (NA)
39 [4]. Currently, 18 HA(H1-H18) and 11 NA (N1-N11) subtypes have been identified in IAVs
40 [5]. Aquatic birds are considered to be the natural reservoirs of IAVs and the source of all
41 IAVs in other animals such as human, poultry, wild birds, swine, equine and so on [6-8].
42 Widespread hosts, the lack of proofreading activity of the viral polymerase during
43 genome replication and the segmented nature of genome enable IAVs rapidly evolve
44 though the accumulation of mutations and genome reassortment [9, 10], leaving an
45 increasing evolution diversity of the IAV and constantly emerged novel IAVs [11], which
46 poses a serious threat to human lives and economic development.

47 Different viruses may have evolved various mechanisms to co-opt host processes
48 and suppress host defenses, inducing different infectious phenotypic outcomes. H1N1 is
49 one of the main seasonal IAVs, which are annually recurrent and are mutually infectious
50 among human population[12, 13]. Highly pathogenic (HP) avian influenza virus (AIV)
51 H5N1 have jumped host barriers and caused human infections since 1997 [14].
52 According to the monitoring report from WHO, H5N1 AIVs have resulted in 860 human
53 infections and 453 deaths as of November 1, 2018 [15]. Constantly reported sporadic
54 human infectious with H5N1 and its high mortality warn us that H5N1 AIV may cause
55 serious epidemic worldwide. It is necessary to improve the epidemiological surveillance
56 and the research of pathogenic mechanism on these important subtypes of influenza
57 viruses.

58 Both H1N1 and H5N1 influenza viruses have posed tremendous health threats in

59 many regions worldwide, but their transmissibility, virulence and fatality are quite
60 different [16]. Although characterized by high transmissibility, the virulence and fatality
61 of the H1N1 influenza virus are relatively low. The reverse holds true for H5N1 influenza.
62 With a fatality rate that exceeds 60%, it is known to cause severe damage to the human
63 respiratory system, but the virus is not presently capable of efficient transmission from
64 human to human.

65 An important step to understand the pathogenesis of the pathogens is to
66 investigate the protein-protein interactions between the host and pathogens. Influenza
67 A viruses infect hosts through complex mechanisms, among which direct binary
68 interactions between influenza A viral proteins and host proteins play an important role
69 during the process influenza viruses invade hosts. The innate immune system is the first
70 barrier to prevent pathogen invasion. The NF-κB signaling pathway is an important part
71 of the innate immune system, which plays a key role in the induction of the innate
72 immune response against viral infection. The important role of NF-κB signaling pathway
73 in resisting virus invasion makes it a hot topic in current research [17-20]. In mammals,
74 there are two major NF-kB signaling pathways: the canonical NF-kB pathway and the
75 noncanonical NF-kB pathway [21, 22]. Under the stimulation of pathogenic microbial
76 infections or their induced inflammatory factors, NF-κB is normally activated by classical
77 signaling pathways [23]. Briefly, NF-κB dimers are retained in the cytoplasm by binding to
78 inhibitory IκB proteins, binding of certain ligands and cell surface receptors recruits
79 downstream adaptor proteins, then they recruit IKK complexes and lead to the activation

80 of IKK complexes [23, 24]. IKK complexes, especially the IKKβ subunit, phosphorylate two
81 serine residues of the IκB protein, resulting in ubiquitination and proteasome-dependent
82 degradation of the IκB protein, which releasing NF-κB into the nucleus to activate
83 downstream gene transcription [25, 26]. The non-canonical NF-κB signaling pathway
84 mainly activates the signal molecule NIK kinase, which in turn phosphorylates a complex
85 containing two IKKα subunits [27]. The IKKα complex phosphorylates and cleaves p100,
86 thereby promoting the form of p52/RelB dimerization which then activates transcription
87 of specific genes [28, 29].

88 Diverse bacterial and viral pathogens target NF-κB signaling pathway to evade host
89 immune defenses. Some viruses suppress NF-κB activation to dampen the host immune
90 responses to maintain latency [30]. For example, human bocavirus (HBoV) proteins NS1
91 and NS1-70 [31], the mumps virus (MuV) small hydrophobic protein (SH) [32] and
92 Molluscum contagiosum virus (MCV) protein MC005 [33] inhibit TNF-α-mediated
93 activation of NF-κB by reducing the phosphorylation of IKKβ, IκBα, and p65 as well as the
94 translocation of p65 into the nucleus. Some viral pathogens activate NF-κB for viral gene
95 expression, replication and spread. For example, the K15 protein of KSHV and the early
96 protein Nef of most primate lentiviruses enhances NF-κB activation to initiate proviral
97 transcription [34, 35]. Influenza A viruses can also interfere with antiviral responses by
98 regulating the NF-κB signaling pathway. Studies have shown that influenza virus proteins
99 interact with NF-κB to promote viral replication. For example, the IAV NS1 protein
100 specifically inhibits IKK-mediated NF-κB activation and production of the NF-κB induced

101 antiviral genes by physically interacting with IKK through the C-terminal effector domain
102 [36].

103 In this paper, we used the luciferase assay and the Co-immunoprecipitation
104 experiment to study how the NP and NA viral proteins of both HIN1 and H5N1 interact
105 with NF-κB signaling pathway. We found that the H1N1 NP has little impact on the
106 TNF-α-induced NF-κB transcriptional activation while the H5N1 NP inhibits the
107 TNF-α-induced NF-κB transcriptional activation by coimmunoprecipitating with IKKα.
108 Furthermore, the H5N1 NP suppresses the phosphorylation of IκBα, IKKα as well as the
109 translocation of p65 into the nucleus of infected A293 cells. We also found that the
110 H5N1 NA actives the IL-1β-induced NF-κB transcriptional activation by
111 coimmunoprecipitating with TAB2, while H1N1 NA inhibits the IL-1β-induced NF-κB
112 transcriptional activation by coimmunoprecipitating with IKKβ.

113 Then, we used a yeast two-hybrid approach to generate an influenza-host
114 protein-protein interaction network for a highly pathogenic avian H5N1 influenza virus
115 A/Goose/Jilin/hb/2003. In order to investigate the pathogenic conservations and
116 distinctions for different influenza virus subtypes, we introduced two other previously
117 reported influenza-host PPI networks (the H1N1 strain A/PR/8/34 and the H3N2 strain
118 A/Udorn/72) [37]. We systematically mapped and compared the pathogen-host protein
119 interactions between proteins from three strains of different influenza A virus subtypes
120 and human proteins. By identifying shared and distinct host-pathogen protein
121 interaction patterns for these influenza A viruses, we enlightened how they exploit

122 distinctive strategies to subvert cellular pathways toward disease progression.

123 Results

124 **1 H5N1 NP and H1N1 NP have different impacts on TNF-α-induced NF-κB activation.**

125 Nucleoprotein (NP) is an important component of ribonucleoprotein (RNP) complex
126 playing crucial role in influenza virus life cycle together with other polymerase proteins
127 (PB1 and PA). At the onset of replication, NP functions as an adapter between the virus
128 and host cell processes to maintain the viral genome integrity. After release of RNP into
129 the cell cytoplasm, NP interacts with many macromolecules of cellular origin to facilitate
130 transcription, replication and translation of viral genome [6, 38].

131 TNF-α plays an important role in host defense against viral infection. During the IAV
132 infection, the elevated level of TNF-α is detected in serum. To determine the impact of
133 NP on the activation of the NF-κB pathway, we evaluated the impact of the H5N1 and
134 H1N1 NP coding sequence on NF-κB activation by using an NF-κB promoter reporter
135 system (Fig. 1A, Fig. 1B). Those results show that H5N1 NP inhibits TNF-α-induced NF-κB
136 transcriptional activation while the H1N1 NP has little impact on it, which confirming
137 that H5N1 NP has an inhibitory effect on NF-κB signaling pathway, but the specific site
138 and mechanism remain unclear. Then we searched for potential targets through the
139 luciferase assay. Those results (Fig. S1) show that the potential targets of H5N1 NP on
140 the NF-κB signaling pathway are IKKα and the complex of TAK. To decide the exact target

141 of H5N1 NP, we investigated whether the H5N1 NP protein physically interacts with IKKα
142 and the complex of TAK by the essay of Co-Immunoprecipitation. The results (Fig. 1C, Fig.
143 1D) show that there are protein-protein interactions between H5N1 NP and TAK1 and
144 IKKα in cells, indicating that the H5N1 NP inhibits the NF-kB signaling pathway by
145 targeting TAK1 and IKKα.

146 We then examined whether H5N1 NP can modulate its activation. The activation of
147 IKKα by phosphorylation is required to the phosphorylation of IκB. We tested whether
148 H5N1 NP played a role in inhibiting the phosphorylation of the IKKα. The results show
149 that the level of phosphor-IKKα in empty vector-transfected cells was more than that in
150 H5N1 NP-expressing cells (Fig. 1E), indicating that the H5N1 NP protein suppresses
151 TNF-α-mediated IKKα phosphorylation.

152 **2 H5N1 NA and H1N1 NA have different impacts on IL-1β-induced NF-κB activation.**

153 Neuraminidase（NA）is an integral membrane glycoprotein and a second major
154 surface antigen of the virion. NA cleaves terminal sialic acid from glycoproteins or
155 glycolipids. Thus, it functions to free virus particles from host cell receptors, to permit
156 progeny virions to escape from the cell in which they arose, and so facilitate virus spread
157 [6, 10].

158 We studied the differences in the impacts of the NA proteins of H5N1 and H1N1 NA
159 on the NF-κB signaling pathways using a similar method through an NF-κB promoter
160 reporter system. Reporter plasmid p NF-κB-luc and internal control plasmid pRL-TK,
161 together with pH5N1 NA and pH1N1 NA or empty vector, were cotransfected into 293T

162 cells. At 24h post-transfection, cells were mock-treated or treated with human IL-1β for
163 6h. The H5N1 NA clone significantly promoted IL-1β-stimulated NF-κB promoter activity
164 (Fig. 2A) while H1N1 NA has inhibitory impact on it (Fig. 2B). This result indicates that
165 H5N1 NA promotes IL-1β-induced NF-κB transcriptional activation while H1N1 NP
166 inhibits it.

167 The potential target of H5N1 NA on the NF-κB signaling pathway is the complex of
168 TAK (Fig. S2) while the potential targets of H1N1 NA on the NF-κB signaling pathway are
169 TAB2 and IKKβ (Fig. S3). Then we also studied the difference of interaction sites between
170 H5N1 NA and H1N1 NA with the NF-κB signaling pathway by the essay of
171 Co-Immunoprecipitation. The result is that H5N1 NA interacts with NF-κB signaling
172 pathway at TAB2 (Fig. 2C) while H1N1 NA interact with NF-κB signaling pathway at IKKβ
173 (Fig. 2D), which demonstrate that H5N1 NA and H1N1 NA have different sites that
174 interact with the NF-κB signaling pathway.

175 **3 Identification of Human Factors that Interacts with Influenza Viruses**

176 Then we seek to find out the virus-host relations of different subtypes of IAVs on a
177 larger scale by comparing the influenza-host PPI networks. We used a yeast two-hybrid
178 (Y2H) approach to identify direct binary interactions between proteins of an H5N1 strain
179 and human proteins. One hundred and seventy-seven pairwise interactions between the
180 8 viral proteins and the 140 human proteins were detected. (Fig. 3, Table S1).

181 Two previous reported influenza-host PPI network [37] were introduced in this
182 paper for comprehensively comparison of networks. The first network contains 135

183 pairwise interactions between the H1N1 strain A/Human/PR/8/34 ("PR8") and 87 human
184 proteins, and the second network covers 81 pairwise of interactions between the H3N2
185 strain A/Human/Udorn/72 ("Udorn") and 66 human proteins. Both of these two
186 pathogen-host networks were collected by using the Y2H approach. Our comparison and
187 network mapping are based on these three influenza-host PPI networks.

188 We generated a human protein-protein interaction network based on the BioGRID
189 interaction database [39], which contains 127950 interactions among 14924 human
190 proteins (only the physical binary interactions are selected). We define the proteins in
191 the human interaction network that directly interact with the influenza viruses as N1
192 proteins, and first neighbors of the N1 proteins are defined as N2 proteins. To quantify
193 the importance or the essentiality of proteins in the human PPI network, we use the
194 degree centrality and a new centrality measurement (named as NC) based on edge
195 clustering coefficient [40].

196 The network topological analysis shows that the viral proteins have a greater
197 average degree in the influenza-host interaction network compared to that of human
198 proteins in the human interaction network (Table 1). The N1 proteins and N2 proteins of
199 influenza viruses on average interact with more proteins in the human interaction
200 network than the others.

201 Based on the other centrality measure NC, both the N1 and N2 proteins of all the
202 three stains of influenza viruses score higher than the average score of the proteins in
203 the human interaction network, especially the N1 proteins of the H5N1 virus. In [41], the

authors proposed that pathogens may have evolved to interact with human proteins that are hubs (those involved in many interactions) or bottlenecks (those central to many pathways) [42] to disrupt key proteins in complexes and pathways. Our results also support this hypothesis. The connection patterns above hold for all the three influenza-host interaction networks, which implying that an influenza virus as a very compacted pathogen has to maximum its function by interacting with many important proteins in the hosts.

4 Different subtypes of influenza viruses show different virus-host interacting patterns

Although all the three strains of influenza viruses share many topological characteristics in the influenza-host interaction network, the direct interactors (N1 proteins) of different subtypes of influenza viruses do not actually overlap much (Fig. 4A). Only 6 proteins (Table 2) are shared by all the three strains as direct interactors. Table 2 has listed all the 6 proteins and their viral interactors of different subtypes of influenza viruses. Although all the three strains of influenza viruses target directly at these six human proteins, the viral proteins they used are different. For example, the viral interactor of the TRAF1 protein in the H5N1 strain is PB1, while the PR8 strain uses two different viral proteins (NP, PB2) to interact with TRAF1.

Despite the three influenza viruses share few direct interactors, they have much more in common at the N2 protein level (Figure 4B). In total, there are 829 proteins shared by all the three influenza viruses.

We then mapped the host interactors to pathways that may involve in the infection of influenza viruses. After filtering, four classes of pathways in the KEGG database [43] were considered: the immune system pathways, the cell growth and death pathways, the signal transduction pathways and pathways about inflammation. In total, sixty-five pathways were selected (Table S2).

Fifty-five out of sixty-five pathways have shown to be in contact with one or more strains of influenza viruses (Fig. 5), which prove the efficiency of our selection in canonical pathways to consider. These include some previously reported pathways that are related to influenza infection such as NF-kB, apoptosis, Wnt/β-catenin and MAPK pathways. There are twenty-two out of sixty-five pathways shown to be involved in the infection of all the three strains of influenza viruses.

Different subtypes of influenza viruses, though have few direct host interactors in common, show much in common at the indirect interactor level (N2 proteins) and the targeted pathway level. This fact implies that influenza viruses infect hosts by targeting functional modules or protein complex in the host PPI network rather than focusing on specific proteins to bind. More details of the relationships between influenza viruses and host pathways show that different influenza viruses use different viral proteins to target different host proteins in the pathways (Table S2).

Discussion

During the course of an influenza virus infection, viral proteins interact with an array

245 of host proteins and pathways, which defines the viral-host relationships. Different
246 viral-host relationships lead to different pathogenesis of the virus. Human may be
247 infected by several subtypes of influenza viruses, including H1N1, H3N2, H5N1, H7N9,
248 H9N2. However, the symptoms of these infections vary from mild to severe illness. In
249 this paper, we tried to uncover the subtype-specific viral-host relationships by examining
250 the effects of viral proteins on the NF-κB signaling pathways. Furthermore, we
251 comprehensively compared the influenza-host PPI networks of three strains of virus in
252 different subtypes, including one that we constructed using the Y2H approach. The
253 results imply that different subtypes of influenza A virus use different viral proteins to
254 target on different human proteins and human cellular pathways, leading to different
255 pathogenic phenotypes.

256 NF-κB is critical for innate immune defense to cope with invading microbial
257 pathogens. Once there are viruses invading organisms, the host will manipulate the
258 NF-κB signaling pathway to clean up them. To survive, the microbial pathogens have
259 evolved various strategies to make use of the NF-κB signaling pathway to escape the
260 innate immune of the host. Influenza viruses can also manipulate the NF-κB signaling
261 pathway to promote their survival and replication. Interaction between influenza virus
262 and NF-κB signaling pathway is essential for its pathogenesis.

263 H1N1 and H5N1 influenza viruses both have the nucleoprotein and neuraminidase,
264 but their transmissibility, virulence and fatality are quite different [16]. Through our
265 studies, we found H5N1 NP inhibits TNF-α-induced NF-κB transcriptional activation while

266 the H1N1 NP has little impact on it and H5N1 NA promotes IL-1β-induced NF-κB
267 transcriptional activation while H1N1 NP inhibits it. The results show subtype-specific
268 characters of the influenza-host protein interactome, which may response for the
269 specific pathogenic mechanisms of different subtypes of influenza viruses.

270 We systematically compared and mapped the influenza-host PPI network of three
271 stains of influenza viruses. Human interactors of different subtypes of influenza viruses
272 have shown to be of similar network topological parameters in the human PPI network.
273 Unlike the other two strains of viruses (PR8, Udorn), which are human-hosted, the highly
274 pathogenic avian H5N1 influenza virus used in this paper is originally an avian influenza
275 virus. This may explain the obvious differences in direct interactors and affected
276 pathways. The H5N1 strain tends to have more interactions with human proteins and
277 affect more cellular pathways than the other two strains. This implies that when avian
278 influenza viruses managed to cross the host barriers infect human, the interactions
279 between them are usually intense. However, for the viruses that have been circulating
280 for a long time in human, like H1N1 and H3N2 influenza viruses, the interactions
281 between the invader and the host become weaker.

282 The conservative patterns for all the three strains of influenza viruses at the N2
283 protein level and the pathway level highlight that pathogens have some common
284 attacking patterns to infect human. Some pathways that are related to all the three
285 strains of viruses are selected. The viral proteins employed to target at these conserved
286 pathways are different for different viruses, suggesting more subtype-specific pathogenic

287 mechanisms need more research work.

288 Materials and Methods

289 1. Antibodies and Reagents.

290 Antibodies for IκBα, α-Tubulin, Flag, HA, MyC, phospho-IκBα and phospho-IKKα
291 were purchased from Cell Signaling. Cell culture products were from Invitrogen and all
292 other chemicals were Sigma-Aldrich products unless noted.

293 2. Cell Culture, Transfection, and Luciferase Reporter Assays.

294 293T (human embryonic kidney) cells were cultured in Dulbecco's modified Eagle's
295 medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum
296 (FBS, Gibco), 2mM L-glutamine, 100U/mL penicillin, and 100mg/mL streptomycin.
297 Transient transfections were performed with Lipofectamine 2000 (Invitrogen) according
298 to the manufacturer's instructions.

299 Luciferase activity was determined by using the dual luciferase assay kit (Promega)
300 according to the manufacturer's instructions.

301 3. Immunoprecipitation and Immunoblotting

302 Immunoprecipitation was performed using IP buffer (1% NP40, 50mM Tris-HCl [pH
303 7.5], 150mM NaCl, and CompleteTM protease inhibitor cocktail-EDTA (Roche)). Whole
304 cell extracts were prepared after transfection and incubated with indicated antibodies
305 together with Protein A/G beads (Roche) overnight. Beads were then washed 4 times

with IP buffer, and immunoprecipitates were eluted with SDS loading buffer (TransGen Biotech) and resolved in SDS-PAGE gels. The proteins were transferred to PVDF membrane (Bio-Rad) and further incubated with the indicated antibodies. The antigen-antibody complexes were visualized by the ImmubilonTM chemiluminescent detection kit (Millipore).

4.Statistical Analysis.

Analyses were done with the statistical software SAS/STAT. Data analysis over time was undertaken by repeated-measures analysis with SAS/STAT. Differences were considered statistically significant if the P value was <0.05.

5.Human Protein-Protein Interaction Network

We use all the protein-protein interaction for homo sapiens in the BioGRID interaction database. The database version is 3.2.106. We choose all the physical interactions in the database, while the genetic interactions are excluded. In total, the PPI network contains 127950 interactions among 14924 human proteins.

6.Protein Centrality Analyses

Based on the human PPI network constructed, we calculated the degree of each node in the network. And the NC centrality based on edge clustering coefficient are calculated by using the following equation:

$$NC(v) = \sum_{u \in N_v} \frac{z_{v,u}}{\min(d_v - 1, d_u - 1)} \quad [40],$$

where $z_{v,u}$ denotes the number of triangles that include the edge actually in the

network, d_u and d_v are degrees of node u and node v, and N_v denotes the set of all neighbors of node v.

7.KEGG Pathways

We download the KGML files at the KEGG pathway database for the pathways belongs to the following categories: immune system pathways, the cell growth and death pathways and the signal transduction pathways and pathways about inflammation. Then, we parse these KGML files by using python programs to obtain the genes that participated in each pathway.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31800136), Beijing Nova Program (Z171100001117120), Independent Research Project from State Key Laboratory of Pathogen and Biosecurity (SKLPBS1807) and Military Medical Science and Technology Youth Development Program (18QNP037).

Author contributions

Hongguang Ren, Long Liang and Junjie Yue conceived and supervised the study. Ting Song and Hongguang Ren designed the experiments. Yujie Wang, Ting Song and Kaiwu Li performed the experiments. Hongguang Ren and Yuan Jin analyzed the data. Hongguang Ren and Yujie Wang wrote the manuscript. Yujie Wang and Ting Song contributed equally to this work.

Reference

1. Nelson, M.I. and A.L. Vincent, *Reverse zoonosis of influenza to swine: new perspectives on the human-animal interface.* Trends in microbiology, 2015. **23**(3): p. 142-153.
2. Gerber, M., et al., *Selective packaging of the influenza A genome and consequences for genetic reassortment.* Trends Microbiol, 2014. **22**(8): p. 446-55.
3. Skehel, J.J. and A.J. Hay, *Influenza Virus Transcription.* Journal of General Virology, 1978. **39**(1): p. 1-8.
4. Lamb, R.A. and R.M. Krug, *In Fields Virology 4th edn*, ed. B.N. eds Fields, Knipe, D. M. & Howley, P. M. 2001: Lippincott-Raven, Philadelphia.
5. Tong, S., et al., *New World Bats Harbor Diverse Influenza A Viruses.* PLOS Pathogens, 2013. **9**(10): p. e1003657.
6. Webster, R.G., et al., *Evolution and ecology of influenza A viruses.* Microbiological reviews, 1992. **56**(1): p. 152-179.
7. Horimoto, T. and Y. Kawaoka, *Influenza: lessons from past pandemics, warnings from current incidents.* Nature Reviews Microbiology, 2005. **3**: p. 591.
8. Zhou, J., et al., *Characterization of the H5N1 highly pathogenic avian influenza virus derived from wild pikas in China.* J Virol, 2009. **83**(17): p. 8957-64.
9. Westgeest, K.B., et al., *Genomewide analysis of reassortment and evolution of human influenza A(H3N2) viruses circulating between 1968 and 2011.* J Virol,

365 2014. **88**(5): p. 2844-57.

366 10. Petrova, V.N. and C.A. Russell, *The evolution of seasonal influenza viruses.* Nat
367 Rev Microbiol, 2018. **16**(1): p. 47-60.

368 11. Su, S., et al., *Epidemiology, Evolution, and Recent Outbreaks of Avian Influenza*
369 *Virus in China.* Journal of Virology, 2015. **89**(17): p. 8671.

370 12. Reid, A.H., et al., *Origin and evolution of the 1918 "Spanish" influenza virus*
371 *hemagglutinin gene.* Proceedings of the National Academy of Sciences, 1999.
372 **96**(4): p. 1651.

373 13. Smith, G.J.D., et al., *Origins and evolutionary genomics of the 2009 swine-origin*
374 *H1N1 influenza A epidemic.* Nature, 2009. **459**: p. 1122.

375 14. Yuen, K.Y., et al., *Clinical features and rapid viral diagnosis of human disease*
376 *associated with avian influenza A H5N1 virus.* Lancet, 1998. **351**(9101): p. 467-71.

377 15. WHO, *Cumulative number of confirmed human cases for avian influenza A(H5N1)*
378 *reported to WHO.* 2018. **1 November 2018**.

379 16. Simon, P.F., et al., *Highly Pathogenic H5N1 and Novel H7N9 Influenza A Viruses*
380 *Induce More Profound Proteomic Host Responses than Seasonal and Pandemic*
381 *H1N1 Strains.* Journal of Proteome Research, 2015. **14**(11): p. 4511-4523.

382 17. Hayden, M.S. and S. Ghosh, *Shared principles in NF-kappaB signaling.* Cell, 2008.
383 **132**(3): p. 344-62.

384 18. Kobayashi, T. and R. Kageyama, *Dynamic advances in NF-kappaB signaling*
385 *analysis.* Sci Signal, 2009. **2**(81): p. pe47.

386 19. Shih, R.-H., C.-Y. Wang, and C.-M. Yang, *NF-kappaB Signaling Pathways in*
387 *Neurological Inflammation: A Mini Review.* Frontiers in Molecular Neuroscience,
388 2015. **8**: p. 77.
389 20. Hayden, M.S. and S. Ghosh, *Signaling to NF-κB.* Genes & Development, 2004.
390 **18**(18): p. 2195-2224.
391 21. Zheng, J.W., et al., *NF-kappaB/P65 signaling pathway: a potential therapeutic*
392 *target in postoperative cognitive dysfunction after sevoflurane anesthesia.* Eur
393 Rev Med Pharmacol Sci, 2017. **21**(2): p. 394-407.
394 22. Sun, S.-C., *The non-canonical NF-κB pathway in immunity and inflammation.*
395 Nature Reviews Immunology, 2017. **17**: p. 545.
396 23. Vallabhapurapu, S. and M. Karin, *Regulation and Function of NF-κB Transcription*
397 *Factors in the Immune System.* Annual Review of Immunology, 2009. **27**(1): p.
398 693-733.
399 24. Denk, A., et al., *Activation of NF-κB via the IκB Kinase Complex Is Both Essential*
400 *and Sufficient for Proinflammatory Gene Expression in Primary Endothelial Cells.*
401 Journal of Biological Chemistry, 2001. **276**(30): p. 28451-28458.
402 25. Durand, J.K. and A.S. Baldwin, *Chapter Three - Targeting IKK and NF-κB for*
403 *Therapy*, in *Advances in Protein Chemistry and Structural Biology*, R. Donev,
404 Editor. 2017, Academic Press. p. 77-115.
405 26. Kondylis, V., et al., *The interplay of IKK, NF-kappaB and RIPK1 signaling in the*
406 *regulation of cell death, tissue homeostasis and inflammation.* Immunol Rev,

407 2017. **277**(1): p. 113-127.

408 27. Brightbill, H.D., et al., *Conditional Deletion of NF-κB–Inducing Kinase (NIK) in*
409 *Adult Mice Disrupts Mature B Cell Survival and Activation.* The Journal of
410 Immunology, 2015. **195**(3): p. 953.

411 28. Duncan, M.C., et al., *Bacterial internalization is required to trigger NIK-dependent*
412 *NF-κB activation in response to the bacterial type three secretion system.* PLOS
413 ONE, 2017. **12**(2): p. e0171406.

414 29. Cildir, G., K.C. Low, and V. Tergaonkar, *Noncanonical NF-kappaB Signaling in*
415 *Health and Disease.* Trends Mol Med, 2016. **22**(5): p. 414-429.

416 30. Rahman, M.M. and G. McFadden, *Modulation of NF-kappaB signalling by*
417 *microbial pathogens.* Nat Rev Microbiol, 2011. **9**(4): p. 291-306.

418 31. Liu, Q., et al., *Human Bocavirus NS1 and NS1-70 Proteins Inhibit*
419 *TNF-alpha-Mediated Activation of NF-kappaB by targeting p65.* Sci Rep, 2016. **6**:
420 p. 28481.

421 32. Franz, S., et al., *Mumps Virus SH Protein Inhibits NF-kappaB Activation by*
422 *Interacting with Tumor Necrosis Factor Receptor 1, Interleukin-1 Receptor 1, and*
423 *Toll-Like Receptor 3 Complexes.* J Virol, 2017. **91**(18).

424 33. Brady, G., et al., *Molluscum Contagiosum Virus Protein MC005 Inhibits*
425 *NF-kappaB Activation by Targeting NEMO-Regulated IkappaB Kinase Activation.* J
426 Virol, 2017. **91**(15).

427 34. Brinkmann, M.M., et al., *Modulation of host gene expression by the K15 protein*

428 *of Kaposi's sarcoma-associated herpesvirus.* J Virol, 2007. **81**(1): p. 42-58.

429 35. Dufrasne, F.E., et al., *Modulation of the NF-kappaB signaling pathway by the*
430 *HIV-2 envelope glycoprotein and its incomplete BST-2 antagonism.* Virology, 2018.
431 **513**: p. 11-16.

432 36. Gao, S., et al., *Influenza A virus-encoded NS1 virulence factor protein inhibits*
433 *innate immune response by targeting IKK.* Cell Microbiol, 2012. **14**(12): p.
434 1849-66.

435 37. Shapira, S.D., et al., *A Physical and Regulatory Map of Host-Influenza Interactions*
436 *Reveals Pathways in H1N1 Infection.* Cell, 2009. **139**(7): p. 1255-1267.

437 38. Portela, A.n. and P. Digard, *The influenza virus nucleoprotein: a multifunctional*
438 *RNA-binding protein pivotal to virus replication.* Journal of General Virology,
439 2002. **83**(4): p. 723-734.

440 39. Stark, C., et al., *BioGRID: a general repository for interaction datasets.* Nucleic
441 Acids Res, 2006. **34**(Database issue): p. D535-9.

442 40. Wang, J., et al., *Identification of essential proteins based on edge clustering*
443 *coefficient.* IEEE/ACM Trans Comput Biol Bioinform, 2012. **9**(4): p. 1070-80.

444 41. Dyer, M.D., T.M. Murali, and B.W. Sobral, *The landscape of human proteins*
445 *interacting with viruses and other pathogens.* PLoS Pathog, 2008. **4**(2): p. e32.

446 42. Yu, H., et al., *The importance of bottlenecks in protein networks: correlation with*
447 *gene essentiality and expression dynamics.* PLoS Comput Biol, 2007. **3**(4): p. e59.

448 43. Kanehisa, M. and S. Goto, *KEGG: kyoto encyclopedia of genes and genomes.*

Nucleic Acids Res, 2000. **28**(1): p. 27-30.

Figure Legends

Figure. 1 H5N1 NP and H1N1 NP have different impacts on TNF-α-induced NF-κB activation. Fig. 1A and Fig. 1B 293T cells in 24-well plates were cotransfected with 125 ng pNF-κ B-luc, 25 ng pRL-TK and indicated amount of H5N1 NP or H1N1 NP expression plasmid, or empty vector for 24 h. Cells were then mock-treated or treated with TNF-α (10 ng/ml) for 6 h. Reporter activity was determined by dual-luciferase reporter assays. The resultant ratios were normalized to the fold-change value by that of TNF-α -untreated cells cotransfected with empty vector, pNF-κ B-luc and pRL-TK. Fig. 1C 293T cells were cotransfected with indicated amount of H5N1 NP and TAK1 expression plasmid, or empty vector for 24 h. Whole cell extracts were prepared by NP40 lysates and incubated with HA antibodies together with Protein A/G beads for 2 h. Beads were then washed 4 times with IP buffer, and immunoprecipitates were eluted with SDS loading buffer and resolved in SDS-PAGE gels. The proteins were transferred to PVDF membrane and further incubated with the indicated antibodies. Fig. 1D 293T cells were cotransfected with indicated amount of H5N1 NP and IKKα expression plasmid, or empty vector for 24 h. Whole cell extracts were prepared by NP40 lysates and incubated with MyC antibodies together with Protein A/G beads for 2 h. Beads were then washed 4 times with IP buffer, and immunoprecipitates were eluted with SDS loading buffer and resolved in SDS-PAGE gels. The proteins were transferred to PVDF membrane and

469 further incubated with the indicated antibodies. Fig. 1E 293T cells were cotransfected
470 with H5N1 NP or empty vector for 24 h. Cells were then mock-treated or treated with
471 TNF-α (10 ng/ml) for 6 h. The cells were eluted with SDS loading buffer and resolved in
472 SDS-PAGE gels. The proteins were transferred to PVDF membrane and further incubated
473 with the IKKα phosphorylation antibodies.

474 **Figure. 2 H5N1 NA and H1N1 NA have different impacts on TNF-α-induced NF-κB**
475 **activation.** Fig. 2A and Fig. 2B 293T cells in 24-well plates were cotransfected with 125
476 ng pNF-κ B-luc, 25 ng pRL-TK and indicated amount of H5N1 NA or H1N1 NA
477 expression plasmid, or empty vector for 24 h. Cells were then mock-treated or treated
478 with IL-1β (10 ng/ml) for 6 h. Reporter activity was determined by dual-luciferase
479 reporter assays. The resultant ratios were normalized to the fold-change value by that of
480 IL-1β -untreated cells cotransfected with empty vector, pNF-κ B-luc and pRL-TK. Fig.
481 2C 293T cells were cotransfected with indicated amount of H5N1 NA and TAB2
482 expression plasmid, or empty vector for 24 h. Whole cell extracts were prepared by NP40
483 lysates and incubated with MyC antibodies together with Protein A/G beads for 2 h.
484 Beads were then washed 4 times with IP buffer, and immunoprecipitates were eluted with
485 SDS loading buffer and resolved in SDS-PAGE gels. The proteins were transferred to
486 PVDF membrane and further incubated with the indicated antibodies. Fig. 2D 293T cells
487 were cotransfected with indicated amount of H1N1 NA and IKKβ expression plasmid, or
488 empty vector for 24 h. Whole cell extracts were prepared by NP40 lysates and incubated
489 with MyC antibodies together with Protein A/G beads for 2 h. Beads were then washed 4

times with IP buffer, and immunoprecipitates were eluted with SDS loading buffer and resolved in SDS-PAGE gels. The proteins were transferred to PVDF membrane and further incubated with the indicated antibodies.

Figure 3. Influenza-Human PPI interactions of an H5N1 virus. Each cricle represents a protein, green represents viral proteins and red represents host proteins. The interactions between influenza proteins and the human proteins are indicated by lines connecting the circles.

Figure 4. Venn Graphs of (A) N1 proteins and (B) N2 proteins for the three strains of influenza viruses.

Figure 5. Viral proteins of the three strains of influenza viruses are shown with their direct interactors' membership in the selected 65 pathways. Each shaded square in the figure represent the corresponding viral protein in the vertical direction interacts with some human proteins involved in the pathway in the horizontal direction.

Figure. S1 The potential targets of H5N1 NP on the NF-κB signaling pathway are IKKα and the complex of TAK. 293T cells in 24-well plates were cotransfected with 125 ng pNF-κ B-luc, 25 ng pRL-TK and indicated amount of H5N1 NP expression plasmid, or empty vector for 24 h. Cells were cotransfected with TRAF2, TAK1 and TAB1, TAB2, IKKα, IKKβ, respectively. Reporter activity was determined by dual-luciferase reporter assays. The resultant ratios were normalized to the fold-change value by that of TRAF2, TAK1 and TAB1, TAB2, IKKα, IKKβ-untreated cells cotransfected with empty vector, pNF-κ B-luc and pRL-TK.

Figure. S2 The potential target of H5N1 NA on the NF-κB signaling pathway is the complex of TAK. 293T cells in 24-well plates were cotransfected with 125 ng pNF-κB-luc, 25 ng pRL-TK and indicated amount of H5N1 NA expression plasmid, or empty vector for 24 h. Cells were cotransfected with TRAF6, TAK1 and TAB1, TAB2, IKKα, IKKβ, respectively. Reporter activity was determined by dual-luciferase reporter assays. The resultant ratios were normalized to the fold-change value by that of TRAF6, TAK1 and TAB1, TAB2, IKKα, IKKβ-untreated cells cotransfected with empty vector, pNF-κB-luc and pRL-TK.

Figure. S3 The potential targets of H1N1 NA on the NF-κB signaling pathway are TAB2 and IKKβ. 293T cells in 24-well plates were cotransfected with 125 ng pNF-κB-luc, 25 ng pRL-TK and indicated amount of H1N1 NA expression plasmid, or empty vector for 24 h. Cells were cotransfected with TRAF6, TAK1 and TAB1, TAB2, IKKα, IKKβ, respectively. Reporter activity was determined by dual-luciferase reporter assays. The resultant ratios were normalized to the fold-change value by that of TRAF6, TAK1 and TAB1, TAB2, IKKα, IKKβ-untreated cells cotransfected with empty vector, pNF-κB-luc and pRL-TK.

Table S1. Viral-Host protein-protein interactions of the H5N1 virus

Table S2. Relations between the influenza A viruses and the selected pathways.

Figure 1

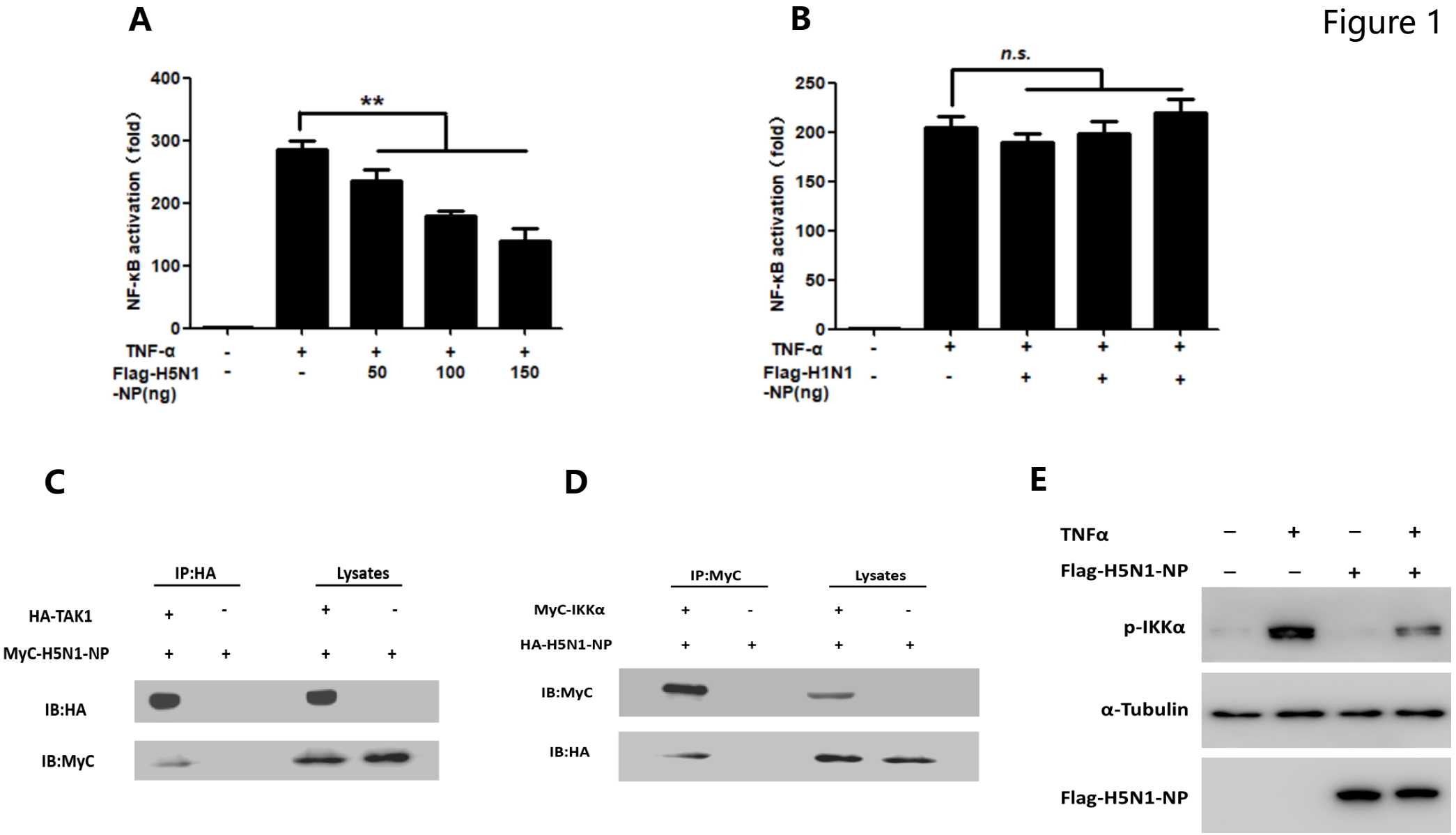

A
NF-κB activation（fold）
400
300
200
100
0
**
TNF-α - + + + +
Flag-H5N1 -NP(ng) - - 50 100 150
B
NF-κB activation（fold）
250
200
150
100
50
0
n.s.
TNF-α - + + + +
Flag-H1N1 -NP(ng) - - + + +
C
IP:HA
Lysates
HA-TAK1 + - + -
MyC-H5N1-NP + + + +
IB:HA
IB:MyC
D
IP:MyC
Lysates
MyC-IKKα + - + -
HA-H5N1-NP + + + +
IB:MyC
IB:HA
E
TNFα – + – +
Flag-H5N1-NP – – + +
p-IKKα
α-Tubulin
Flag-H5N1-NP

Figure 2

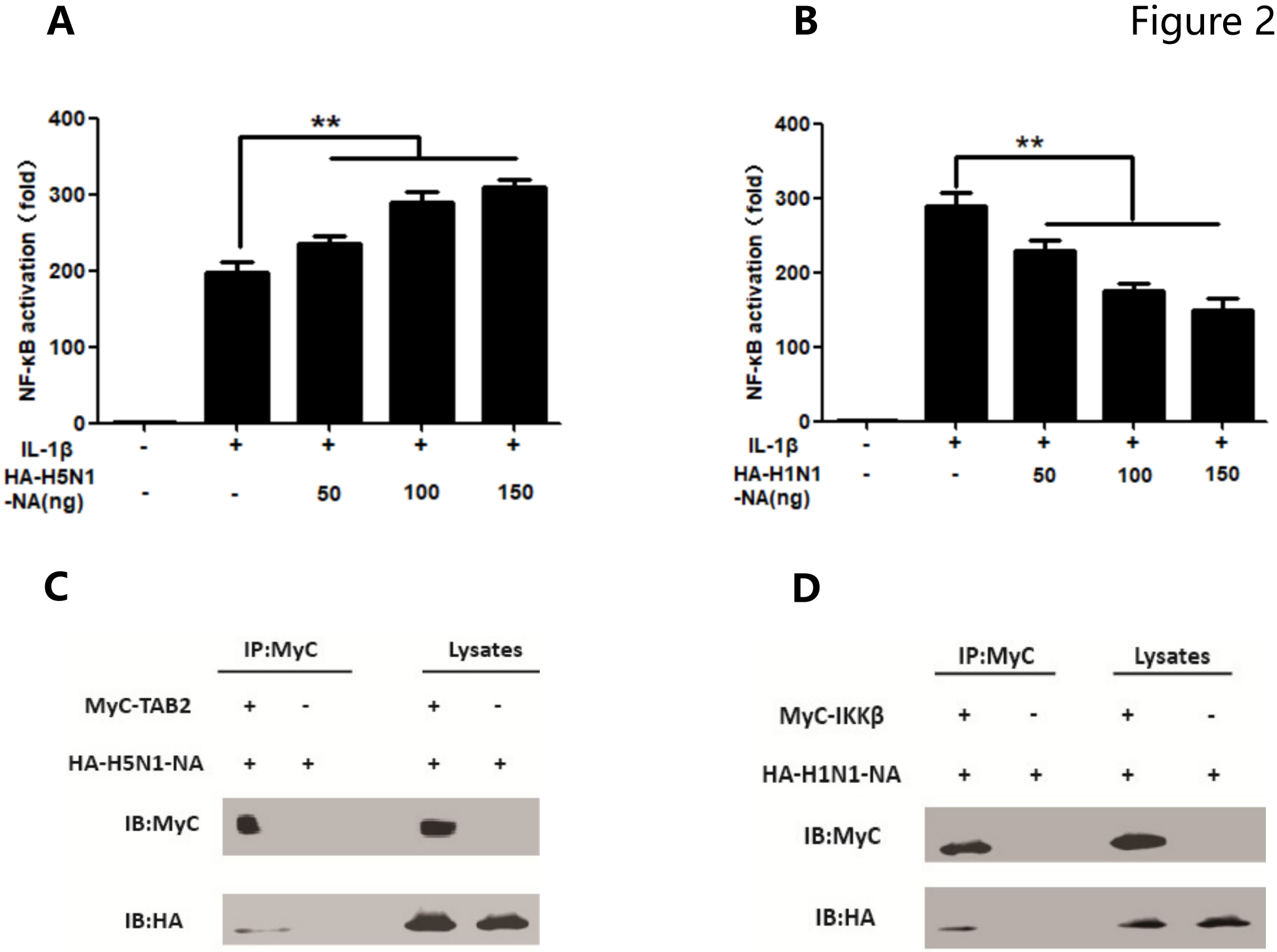
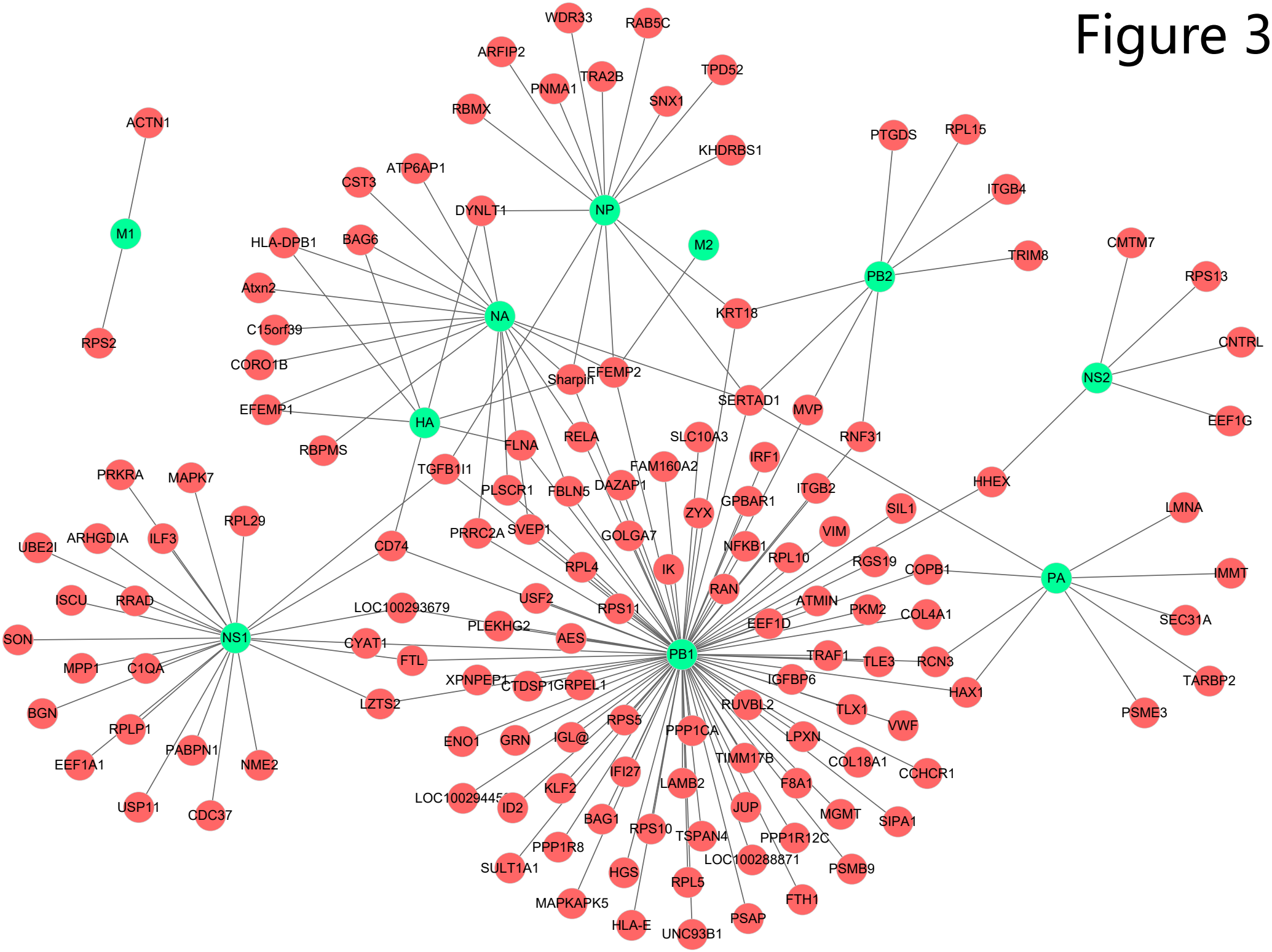
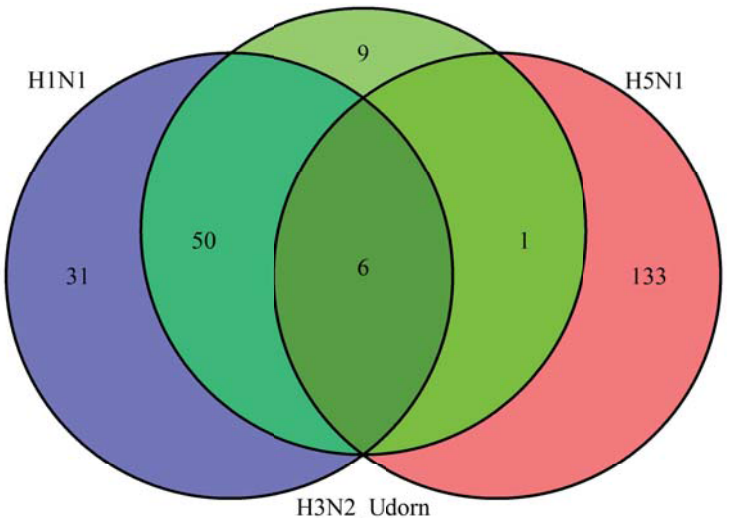

A
B
C
D
NF-κB activation（fold）
400
300
200
100
0
**
IL-1β
HA-H5N1 -NA(ng)
HA-H1N1 -NA(ng)
50
100
150
IP:MyC
Lysates
MyC-TAB2
HA-H5N1-NA
IB:MyC
IB:HA
MyC-IKKβ
HA-H1N1-NA

Figure 3

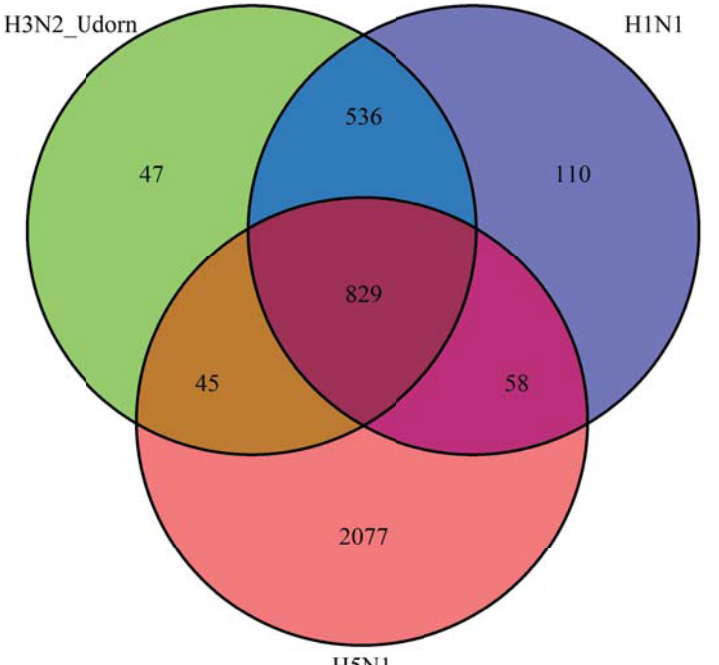


WDR33
RAB5C
ARFIP2
TRA2B
TPD52
PNMA1
SNX1
RBMX
ACTN1
KHDRBS1
PTGDS
RPL15
ATP6AP1
CST3
ITGB4
DYNLT1
NP
M1
HLA-DPB1
BAG6
M2
TRIM8
CMTM7
PB2
RPS13
Atxn2
NA
KRT18
C15orf39
RPS2
CNTRL
CORO1B
Sharpin
EFEMP2
NS2
SERTAD1
MVP
EFEMP1
HA
EEF1G
RELA
SLC10A3
RNF31
RBPMS
FLNA
IRF1
FAM160A2
TGFB1I1
PRKRA
MAPK7
PLSCR1
FBLN5
DAZAP1
ITGB2
HHEX
GPBAR1
ZYX
SIL1
LMNA
RPL29
PRRC2A
SVEP1
GOLGA7
VIM
UBE2I
ARHGDIA
ILF3
CD74
NFKB1
RPL10
RGS19
COPB1
IMMT
RPL4
IK
PA
RAN
ISCU
RRAD
USF2
ATMIN
LOC100293679
RPS11
PKM2
COL4A1
SEC31A
PLEKHG2
EEF1D
SON
NS1
AES
CYAT1
PB1
TRAF1
TLE3
RCN3
MPP1
C1QA
FTL
XPNPEP1
CTDSP1
GRPEL1
IGFBP6
TARBP2
HAX1
LZTS2
RUVBL2
TLX1
BGN
PSME3
RPS5
VWF
RPLP1
PPP1CA
ENO1
GRN
IGL@
LPXN
PABPN1
TIMM17B
COL18A1
EEF1A1
NME2
IFI27
CCHCR1
LAMB2
F8A1
KLF2
LOC100294452
ID2
JUP
MGMT
USP11
CDC37
BAG1
SIPA1
RPS10
TSPAN4
PPP1R12C
PPP1R8
LOC100288871
SULT1A1
HGS
PSMB9
RPL5
FTH1
MAPKAPK5
PSAP
HLA-E
UNC93B1

Figure 4


H1N1
H5N1
H3N2_Udorn
9
31
50
6
1
133

A


H3N2_Udorn
H1N1
H5N1
536
47
110
829
45
58
2077

B

Figure 5

H1N1
H3N2_Udorn
H5N1
Fc gamma R-mediated phagocytosis
Calcium signaling pathway
Phosphatidylinositol signaling system
Pathogenic Escherichia coli infection
Arrhythmogenic right ventricular cardiomyopathy (ARVC)
Antigen processing and presentation
Chemical carcinogenesis
Staphylococcus aureus infection
RIG-I-like receptor signaling pathway
Alzheimer's disease
Hippo signaling pathway
Fc epsilon RI signaling pathway
Prostate cancer
Leukocyte transendothelial migration
Ras signaling pathway
Cell adhesion molecules (CAMs)
Epithelial cell signaling in Helicobacter pylori infection
HIF-1 signaling pathway
Legionellosis
Toxoplasmosis
Proteasome
Complement and coagulation cascades
Inflammatory bowel disease (IBD)
NF-kappa B signaling pathway
Cytokine-cytokine receptor interaction
Non-alcoholic fatty liver disease (NAFLD)
MAPK signaling pathway
Rheumatoid arthritis
p53 signaling pathway
Viral myocarditis
Hematopoietic cell lineage
Asthma
Salmonella infection
Apoptosis
B cell receptor signaling pathway
NOD-like receptor signaling pathway
Phagosome
Tuberculosis
Toll-like receptor signaling pathway
Chemokine signaling pathway
Natural killer cell mediated cytotoxicity
T cell receptor signaling pathway
VEGF signaling pathway
PI3K-Akt signaling pathway
mTOR signaling pathway
Wnt signaling pathway
Notch signaling pathway
Cytosolic DNA-sensing pathway
Shigellosis
Viral carcinogenesis
Graft-versus-host disease
MicroRNAs in cancer
Rap1 signaling pathway
TNF signaling pathway
Systemic lupus erythematosus
ErbB signaling pathway
TGF-beta signaling pathway
Intestinal immune network for IgA production
Cell cycle
HTLV-I infection
Adipocytokine signaling pathway
Hedgehog signaling pathway
Jak-STAT signaling pathway
Amoebiasis
Type I diabetes mellitus
PB2
PB1
PA
HA
NP
NA
M1
M2
NS1
NS2