Bull Math Biol (2013) 75:988–1011
DOI 10.1007/s11538-012-9777-2

Society for Mathematical Biology

ORIGINAL ARTICLE

Dynamics of Influenza Virus and Human Host Interactions During Infection and Replication Cycle

Alex Madrahimov · Tomáš Helikar · Bryan Kowal · Guoqing Lu · Jim Rogers

Received: 24 March 2012 / Accepted: 26 September 2012 / Published online: 19 October 2012

Abstract The replication and life cycle of the influenza virus is governed by an intricate network of intracellular regulatory events during infection, including interactions with an even more complex system of biochemical interactions of the host cell. Computational modeling and systems biology have been successfully employed to further the understanding of various biological systems, however, computational studies of the complexity of intracellular interactions during influenza infection is lacking. In this work, we present the first large-scale dynamical model of the infection and replication cycle of influenza, as well as some of its interactions with the host's signaling machinery. Specifically, we focus on and visualize the dynamics of the internalization and endocytosis of the virus, replication and translation of its genomic components, as well as the assembly of progeny virions. Simulations and analyses of the models dynamics qualitatively reproduced numerous biological phenomena discovered in the laboratory. Finally, comparisons of the dynamics of existing and proposed drugs, our

First and second authors with equal contribution.

Electronic supplementary material The online version of this article (doi:10.1007/s11538-012-9777-2) contains supplementary material, which is available to authorized users.

A. Madrahimov · G. Lu
Department of Biology, University of Nebraska at Omaha, Omaha, NE 68182, USA

A. Madrahimov · T. Helikar (✉) · J. Rogers
Department of Mathematics, University of Nebraska at Omaha, Omaha, NE 68182, USA
e-mail: thelikar@unomaha.edu

B. Kowal
College of Information Technology, University of Nebraska at Omaha, Omaha, NE 68182, USA

J. Rogers
Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68198-5900, USA

results suggest that a drug targeting PB1:PA would be more efficient than existing Amantadin/Rimantaine or Zanamivir/Oseltamivir.

Keywords Influenza A · Systems biology · Dynamical model · Computational modeling · Probabilistic Boolean network

1 Introduction

The emergence and global spread of the pandemic H1N1 2009 influenza virus rapidly infected millions of people worldwide and resulted in more than 18,000 deaths (http://www.cdc.gov/h1n1flu/estimates_2009_h1n1.htm). This called attention to the fact that a complete understanding of influenza infection and the interactions between influenza and its human host remains lacking albeit much has been done in influenza prevention and control. The Influenza A virus evolves rapidly through the accumulation of mutations on the surface (antigenic drift) and through the reassortment of entire gene segments (Webster et al. 1992). Both genetic mutation and genome reassortment give rise to new strains of the virus and hence pose a great challenge in the development of efficient vaccines and drugs.

This challenge is further exacerbated by the biochemical complexity of virus-host interactions and dynamics. Improved understanding of these interactions has the potential to lead to novel targets for the development of drugs and vaccines (Yan 2010). Upon infection influenza virus interacts with a myriad of intracellular molecules in the host, and these interactions play a significant and complex role in the influenza replication cycle (Ludwig et al. 2006; Watanabe et al. 2010). The complexity of virus-host interactions is threefold. First, while the influenza virus produces a mere 10 proteins, these proteins form an extensive network of interactions (Shapira et al. 2009) making the process of identifying the most appropriate targets for antiviral therapy difficult. Second, upon the infection of a host cell by the virus a complex network of immunity response-related biochemical signaling pathways is activated as the host attempts to fight the infection (Oslund and Baumgarth 2011; Song et al. 2011). Third, inside the infected cells, the viral proteins also interact with a large number of host signaling pathways that govern cellular functions under normal (i.e., non-infectious) conditions. These pathways also consist of hundreds of components and form intricate and elaborate networks (Helikar et al. 2008; Shapira et al. 2009). By interconnecting with these biochemical networks, the virus is able to utilize various mechanisms not only to survive in the host, but also replicate in a highly efficient manner (Turan et al. 2004; Yan 2010).

This intricacy, ranging from influenza's rapid evolution to the complex biochemical interactions with its host, impedes effective influenza surveillance and control. In order to fully understand the mechanisms of influenza infections a systems biology approach is required (Forst 2006; Kitano 2002; Trautmann and Sekaly 2011; Yan 2010). Systems biology has the potential to further the understanding of the complex, non-linear biological/biochemical structures that govern the most fundamental functions of living systems. Using computer models of biological structures one can study the theoretical properties of these structures (such as emergent information processing capabilities) as well as employ these models to conduct thousands

of virtual experiments in a fraction of time it takes to conduct experimental studies. This ability to conduct a large number of (virtual) experiments provides a means to weed out "bad" hypotheses that could lead to wasted resources (e.g., money and time) and also generate new hypotheses. However, computational models of influenza-host interactions to better understand the molecular dynamics during influenza infection are lacking. While many mathematical frameworks, ranging from differential equations to discrete models, have been used to model and analyze various biological systems, computational methods to study the dynamics of influenza infection are currently limited to a few works focusing on infections from the cellular level while treating the molecular interactions as a "black box" (e.g. Beauchemin et al. 2005; Hancioglu et al. 2007). To the best of our knowledge, the only other computational model which considers the molecular process of influenza replication cycle is based on ordinary differential equations (Sidorenko and Reichl 2004). Discrete (e.g., Boolean) models have become a popular choice as the computational framework due to their qualitative nature which does not require kinetic parameters for the construction and simulations/analyses of the models (e.g., Morris et al. 2010 and Helikar et al. 2011 review a number of studies, and Rodríguez et al. 2012; Beyer et al. 2011 are recent examples utilizing logical modeling).

Herein we present the first comprehensive dynamical model of the influenza-host interactions during infection of an epithelial cell. The proposed model is based on the Boolean framework in that all species (i.e., nodes) in the model are defined as binary switches that can assume a value of 0 (inactive) or 1 (active) at time t; however, the dynamics of the simulated model are expressed as continuous variables which enables us to capture richer dynamics of the biological system (Helikar and Rogers 2009; Helikar et al. 2008). To interrogate the dynamics of the model we used a software platform, The Cell Collective (http://www.thecellcollective.org; Helikar et al. 2012a), which allows scientists to build computational models in a user-friendly, non-mathematical fashion and quickly simulate the dynamics of the model under hundreds of conditions. Simulations and dynamical analyses of the model highlight the importance and effects of non-linear network structures (such as feedforward and positive feedback loops) on the dynamics of the entry into the host cell and subsequent replication cycle. Furthermore, simulations of the presented model allowed us to visualize the dynamics of the infection when interventions (e.g., via various inhibitors) targeting the host signaling pathways are employed. Specifically, we are able to visualize an enhanced viral uptake by the host cell when receptor tyrosine kinase (RTK) receptors are over-expressed, as well as dampened virus internalization as a result of the inhibition of protein kinase C (PKC) using a specific inhibitor. We also observe that the inhibition of the Raf/Mek/Erk pathway prevents the export of progeny viral particles from the nucleus to the cytoplasm of the host. Finally, simulations of the model predict a more effective target for antiviral therapies, the PB1:PA complex, when compared to two existing drugs (Zanamivir/Oseltamivir and Amantadine/Rimantadine). In the following section is discussed the biology of influenza infection and the virus' interactions with the host, forming the basis for the computational model and simulations discussed in the Results section.

2 The Molecular Biology of Influenza A Infection and Virus-Host Interactions

Influenza virus belongs to the *Orthomyxoviridae* family. To date, three types—A, B, and C—have been identified. The influenza A virus (for simplicity, referred to "Influenza" thereafter) is more pathogenic than B and C viruses and thus becomes the focus of our study. Influenza virus is an enveloped virus containing eight single-stranded negative RNA segments. These segments encode a total of 10 or 11 proteins (depending on the strain) necessary for effective infection of the host, and subsequent replication. These proteins include haemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix proteins 1 and 2 (M1 and M2), nonstructural proteins 1 and 2 (NS1 and NS2), polymerase basic 1 protein (PB1), PB1-F2, polymerase basic 2 protein (PB2), and polymerase acidic protein (PA). A number of these proteins are packaged in the virion. For instance, HA, NA, and M2 are embedded in the envelope of the virion, M1 is found under the membrane, PA, PB1, and PB2 associate with the viral RNA (vRNA) and form a RNA polymerase complex, and NP coats the genome. NS1 and PB1-F2 are not contained in the virion, though they are expressed in the infected host cell (Krumbholz et al. 2011; Payungporn et al. 2010). Influenza infection of a host cell is a multi-step process (Fig. 1): the virus first binds to the host epithelial cell via the interaction of HA with the cell's sialic acid receptors; once bound, the virion is internalized via receptor-mediated endocytosis, upon which its genome is delivered to and released in the host cell's nucleus for transcription and replication (Herz et al. 1981).

Replication in the nucleus is mediated via the released vRNP (PA, PB1, and PB2) and occurs in two steps: the negative sense vRNA is copied to positive sense com-

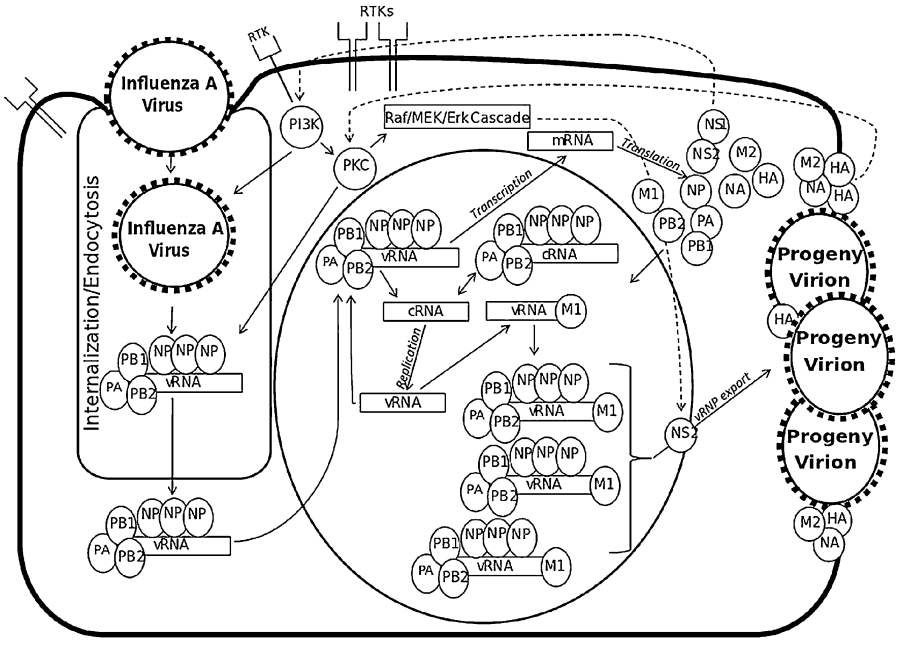

Fig. 1 Biology of the influenza replication cycle. Explanation can be found in the main text

plementary RNA (cRNA) which subsequently serves as a template to transcribe to progeny negative sense vRNA (Engelhardt and Fodor 2006; O'Neill et al. 1998). Newly synthesized vRNAs are subsequently packaged to form active viral ribonucleoproteins (vRNPs) by binding to NP and the RNA polymerase complex (Detjen et al. 1987). Newly generated vRNPs are then exported from the nucleus to cytoplasm with the help of the NS2 and M1 proteins (Boulo et al. 2007). Once the replicated vRNPs are in the cytoplasm new viral particles are formed. The formation of new virions occurs via budding on the apical plasma membrane of the infected cell. In this process a number of the viral proteins play an important role; specifically, the M1 and M2 proteins are important for the budding process as a docking station and the final pinch-off of the virus whereas HA and NA must be present to be incorporated into the envelope of the new virion (Rossman and Lamb 2011).

In addition to the viral proteins, a number of host cellular proteins are involved in the infection process. By connecting into these biochemical networks, the virus is able to utilize various host mechanisms to maximize its replication and survival in the host (König et al. 2010; Watanabe et al. 2010). For example, the viral NS1 protein interacts with hosts' CPSF4 and PABII proteins to inhibit antiviral mRNA processing (Yan 2010). This NS1 protein also stimulates the PI3K/Akt signaling pathway in the infected cell to promote efficient virus replication while inhibiting antiviral response (Yan 2010). The viral PB2 and NP proteins also stimulate apoptosis pathways in the host cell (Turan et al. 2004). In the presented model we focus on the interaction of the viral proteins with the PI3K and Raf/Mek/Erk signaling pathways.

3 Methods

3.1 Our Modeling Technique

The mathematical framework used herein is a hybrid of traditional Boolean networks and Boolean control networks—probabilistic Boolean control networks. As in any type of Boolean networks, all nodes in our model can assume an active (1) or inactive (0) state at any time t. However, in our system, nodes are further classified into two types. As in traditional Boolean networks, the first type of nodes are ones whose activity state is regulated by any number of neighboring nodes in the network. The regulatory mechanisms of these nodes are represented via Boolean functions (e.g., in the form of truth tables or Boolean expressions). Traditional Boolean networks are autonomous—all nodes are regulated by at least one other component in the network (the in-degree of each node is at least 1). However, biological systems are generally stimulated by external factors. For example, signal transduction pathways regulating cell growth are stimulated by external ligands such as growth factors that bind to and activate their receptors that in turn activate the corresponding pathways. Boolean models that account for these external factors are referred to as Boolean Control Networks (Cheng and Qi 2010), an extension of traditional (autonomous) Boolean networks. In addition to the nodes being regulated by other nodes in the network (like in traditional Boolean networks), BCNs contain a subset of nodes which have no regulators. We refer to these nodes as external input species, and are the second

type of nodes included in our model. A number of works utilizing this approach have already been done, for example, in the area of T-cell signaling (e.g., Naldi et al. 2010; Saez-Rodriguez et al. 2007).

Our modeling technique extends these methodologies by introducing a stochastic element—an important feature of biological systems (Abdi et al. 2008; Helikar et al. 2008; Zheng et al. 2010). While BCNs have been used to simulate and analyze the behavior of the corresponding biological systems under external conditions that involve setting the external input species to 0 or 1 for the time of the simulation, in our modeling approach, the 0/1 state of these nodes at time t is determined during simulations via a defined probability. For example, if an external input species is set to "60 %" for a simulation consisting of 100 iterations, the state of the external input species at each time point has a probability of 0.6 of being 1. Further explanation and examples can be found in our recent works (Helikar and Rogers 2009; Helikar et al. 2008).

3.2 Model Construction via the Cell Collective

The Cell Collective (www.thecellcollective.org; Helikar et al. 2012a) is a collaborative modeling platform for large-scale biological systems. The platform allows users to construct and simulate large-scale computational models of various biological processes based on qualitative interaction information using the platform's Bio-Logic Builder (Helikar et al. 2012b). All information about the molecular biology of the influenza virus and its host interactions was retrieved manually from published literature and subsequently entered into the software using the Bio-Logic Builder tool. This tool (automatically) converts this qualitative biochemical information into mathematical expressions in the background. Researchers have the option to export and view these expressions in the form of a logical truth table, Boolean expression, and the Systems Biology Markup Language file from the platform (Boolean rules for each node and works used to devise these rules are listed in the electronic supplementary material). This non-technical creation and representation of the individual interactions in the model make it especially easy for laboratory scientists to contribute to the creation of the model without the need for training in the underlying mathematical formalisms.

The Cell Collective's Knowledge Base component was also used to catalog and annotate all biochemical/biological information for the presented model as mined from the primary literature. Each model species in the Knowledge Base has its own wiki-like page where information on the individual interactions is stored, including bibliography.

3.3 Model Simulations

The Cell Collective platform was also used to perform all computational simulations of the influenza model. While the dynamical model is based on a discrete (i.e., Boolean) formalism, the simulation input and output data are continuous. This was accomplished by converting the digital output of the model simulations to % activity (%ON) which ranges (for each model component) from 0 to

100 % (Helikar and Rogers 2009; Helikar et al. 2008). It is important to note that the % activity does not directly correspond to the biological concentration or any other measurable properties, rather it provides a semi-quantitative measure to describe the activity level of a particular molecular species. To analyze the behavior of different viral or host species under various conditions, each simulation experiment consists of 200 simulations with a different activity level randomly selected for the external nodes in an activity range (0–100 %) defined for the experiment. Each simulation is synchronous (i.e., all nodes are updated simultaneously) and consists of 800 steps. The %ON of the output species is calculated as the fraction of 0's and 1's over the last 300 iterations (Helikar and Rogers 2009; Helikar et al. 2008). As noted previously (Helikar et al. 2008) and from observations of the presented model, the quick settling of the network makes these parameters sufficient to describe the network's "long-term" (e.g., attractor-like) behavior. Quick video tutorials on how to build and simulate models in The Cell Collective are available at http://www.thecellcollective.org. Note that while the simulation engine (ChemChains, Helikar and Rogers 2009) can also conduct asynchronous simulations (different nodes are updated in different times), biological information regarding different timescales in the system modeled herein is currently unavailable, and hence synchronous updates were chosen. As this type of information becomes available, it will be possible to incorporate it in the model and simulations; however, previous studies indicate that asynchronous updating does not result in different steady states (Chaves et al. 2005, 2006).

3.4 External and Initial Simulation Conditions

Dose-response simulations were performed in "controlled" environments. For example, to observe the effects of the viral dose on the replication cycle of the virus, only the node "Viral Dose" was varied for each simulation, while keeping other external input species completely inactivated (at 0 %) or at high levels (90–100 %), depending on the desired experiment and its controls. Specific external conditions are provided in the appropriate figure legends. All simulations are based on the assumption that the host cell is in a quiescent and pre-infected state; hence all nodes are inactive (i.e., 0) at the beginning of the simulation.

4 Results

4.1 The Influenza Model

The influenza virus is composed of 10–11 proteins that are responsible for the infection of the host cell as well as the subsequent replication. Despite the seemingly simple structure of the virus, the ability of the viral proteins to interact with the host cell's machinery make the surveillance and control of influenza infections and pandemics difficult. The virus interacts with different types of host cell with different purposes. For example, infection of an immune cell (e.g., macrophage) results in cell death and weakening the immune system. On the other hand, infected airway epithelial cells are

initially kept alive and controlled so that the virus can replicate more efficiently. The presented model considers the life cycle of influenza infection of an epithelial cell. Hence, processes such as virus-host cell attachment, endocytosis, genome replication and translation, and progeny virion assembly are included. In addition, a number of key host signaling pathways (e.g., Erk and PI3K) known to interact with the virus replication cycle were also included in the model.

As discussed in the *Methods* section, our model is of a qualitative (i.e., Boolean) nature in that any node in the model can be either active or inactive, and the regulatory mechanisms governing the binary state of each node in the model are described with Boolean functions. The interaction graph of the model is visualized in Fig. 2. The nodes in the presented model represent the (i) viral proteins, (ii) intermediate biological molecules and the complexes they form, synthesized in the course of the viral replication cycle such as cRNA, mRNA, vRNP, etc., and (iii) components of the host PI3K and Erk signaling pathway. As can be seen in the interaction graph of the model, the endocytosis pathway of the virus bound to the sialic acid receptor was modeled as a sequence of nodes which represent a different qualitative step in the endocytosis process (e.g., clathrin-coated pit formation, clathrin-coated vesicle, as well as early, maturing, and late endosome). Modeling each of these phases individually provides the ability and flexibility to model effects of additional host signaling pathways on the endocytosis process at different time points. For example, in the following sections we explore at the effects of protein kinase C (PKC) and Erk on replication of the virus. Each segment of the influenza genome acts in an autonomous fashion; that is, each segment is transcribed and replicated individually and hence contributes to the overall production of progeny virions individually. To capture these details in our model we included nodes that represent the vRNP, cRNA, and cRNP for each of the eight genome segments. In addition, nodes representing the mRNA for each viral protein were also included. Our model also captures the dynamics related to localization of some of the aforementioned viral components in the replication cycle of the virus. Specifically, to model the export of newly produced vRNP from the nucleus to the cytoplasm, the model contains two types of vRNP node for each segment: vRNP-M1 complex in nucleus (e.g., vRNP_M1_nuc) and in cytoplasm (e.g., vRNP_M1_cyt). This model allowed us to visualize the dynamics of the vRNP export process during replication (see the following sections with specific examples).

Furthermore, the model has 11 external nodes: the affinity between HA and the host sialic acid receptors (*HA_SialicAcid_Affinity*), virus dose (*Virus_Dose*) representing the amount of infecting virus particles (virions), Mek inhibitor (U0126), protein kinase C (PKC) inhibitor Bisindolylmaleimide I, lipid micro-domain disrupting agent Methyl Beta Cyclodextrin, PI3K inhibitor Wortmannin, and a generic RTK inhibitor *(RTK_Inhibitor).* As discussed in the *Methods* section, the activation level of these nodes can be varied during the simulations in a continuous fashion; that is the activity level can be set to a level between 0 and 100 (or any other range) representing a continuous change in the signal. Together, the model is comprised of 129 nodes (molecular components) and 288 edges (biochemical interactions), representing the largest dynamical model of an influenza-host interaction system.

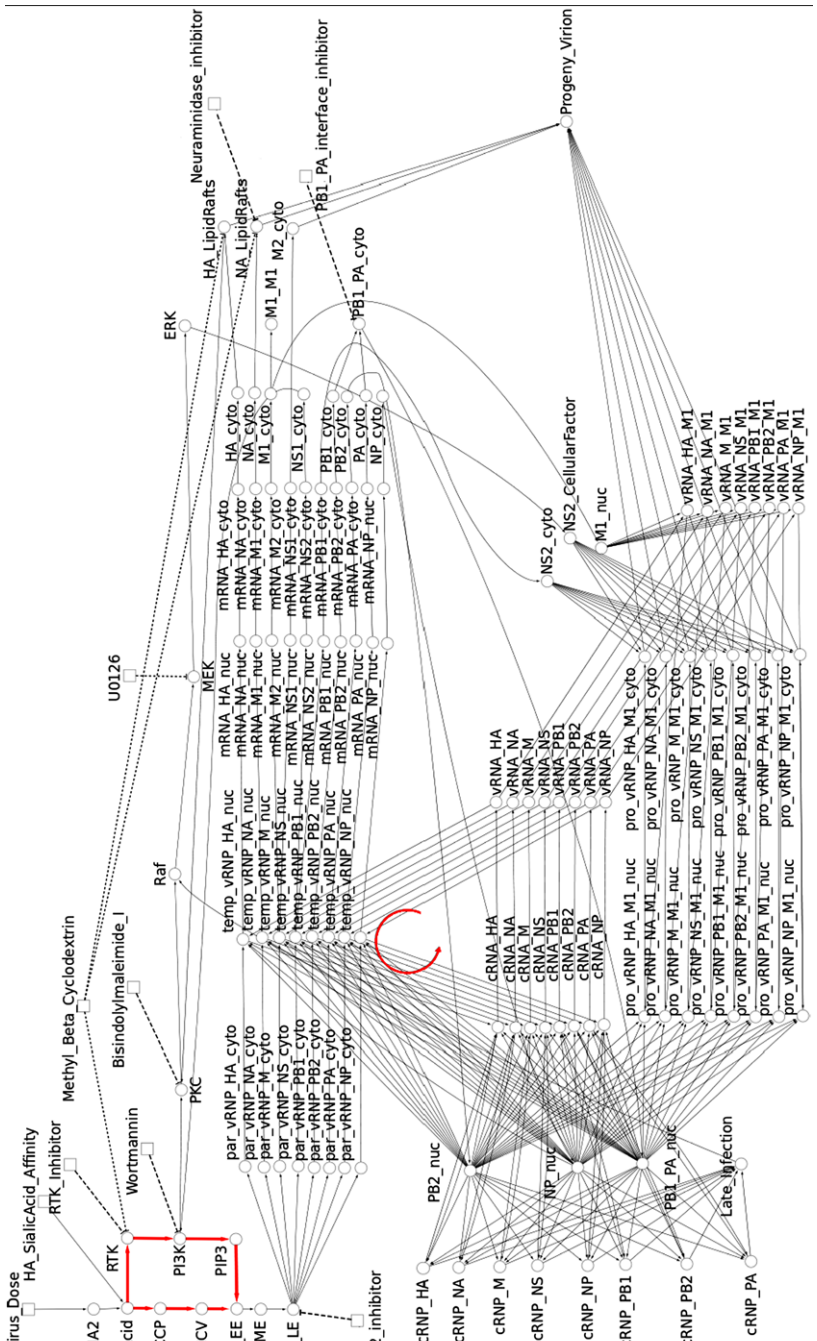

Fig. 2 Interaction graph of the influenza model. *Square nodes* represent external input species whose activity is determined by an assigned probability. *Circled nodes* represent internal nodes whose activity state is determined via a Boolean function. *Normal arrows* represent activation, whereas *blunt arrows* correspond to inhibition. *Red edges* represent the internalization feedforward loop, whereas the *circular arrow* highlights the positive feedback loop of the replication cycle (Color figure online)

4.2 Emergent Dynamics of the Influenza Infection and Replication Regulatory Network

As can be seen from the interaction diagram (Fig. 2), the molecular components governing the process of the influenza infection form a complex regulatory network. In fact, such network-like structure can be found across the biological spectrum, from the biology of yeast (Emmert-Streib and Dehmer 2009) to plants (Umezawa 2011) to human cells (Helikar et al. 2008), to name a few examples. Studies have yielded evidence that these biochemical networks have evolved to perform complex information processing and decision-making tasks in order for the cells to appropriately respond to environmental cues that can often be noisy or even contradictory (Helikar et al. 2008). It has also been suggested that some of these sophisticated functions emerge from the complex interplay between many non-linear architectural features of these networks. Examples of some of the common structures found in many biological processes include positive and negative feedback loops, feedforward loops, and bifans (Ma'ayan et al. 2005). In fact, it turns out that the regulatory network governing the replication cycle of the influenza virus contains a number of these structural features giving rise to non-linear dynamics. In this section we discuss some of the most prominent dynamical properties observed via simulations of the proposed influenza replication network; specifically, we focus on feedforward and positive feedback loops and the dynamics thereof observed in the model.

Feedforward loops have been found in the regulatory networks of mammalian cells (Boyer et al. 2005; Odom et al. 2004) as well in yeast (Lee et al. 2002; Milo et al. 2002) and *E. coli* (Mangan et al. 2003; Shen-Orr et al. 2002). At least eight types of feedforward loop exist (and can be reviewed in Alon 2007; Mangan and Alon 2003). One of the main dynamical properties that arises from feedforward feedback loops (specifically the type illustrated herein) is delayed responses. These feedforward loop-generated delays allow biological systems to filter out noisy or short-lived impulses of signals; instead, a longer and persistent signal is required to overcome the designed threshold in order to activate the target component. As can be seen in the interaction diagram of the influenza-host cell (Fig. 2), a feedforward loop is involved in the regulation of the entry of the virus into the host cell. Specifically, the binding of the infecting virion to the host cell's sialic acid receptor first induces the activation of RTK receptors (e.g., epidermal growth factor receptors) which results in the activation of PI3K and subsequent generation of phosphatidylinositol-3,4,5-triphosphate (PIP3) (Eierhoff et al. 2010). In addition, the binding of the virion to the sialic acid receptor also triggers the internalization (endocytosis) of the receptor-virus complex in the host cell. Before the virus can release its contents in the nucleus of the infected cell, the receptor-virus complex is encapsulated in a clathrin-coated vesicle and subsequently transitioned through early, maturing, and late endosome. However, the formation of early endosome has been proposed to be dependent on PI3K (Ehrhardt et al. 2006). Hence the initial steps of the internalization pathway and the concurrent activation of the RTK/PI3K pathway form a feedforward loop (Fig. 3, Panels A–D). Panels A and B (Fig. 3) also suggest that stimulation of the sialic acid receptor bound virus in clathrin-coated vesicles and PI3K pathway (via RTK receptors), respectively, is positively correlates with the level of infecting viral

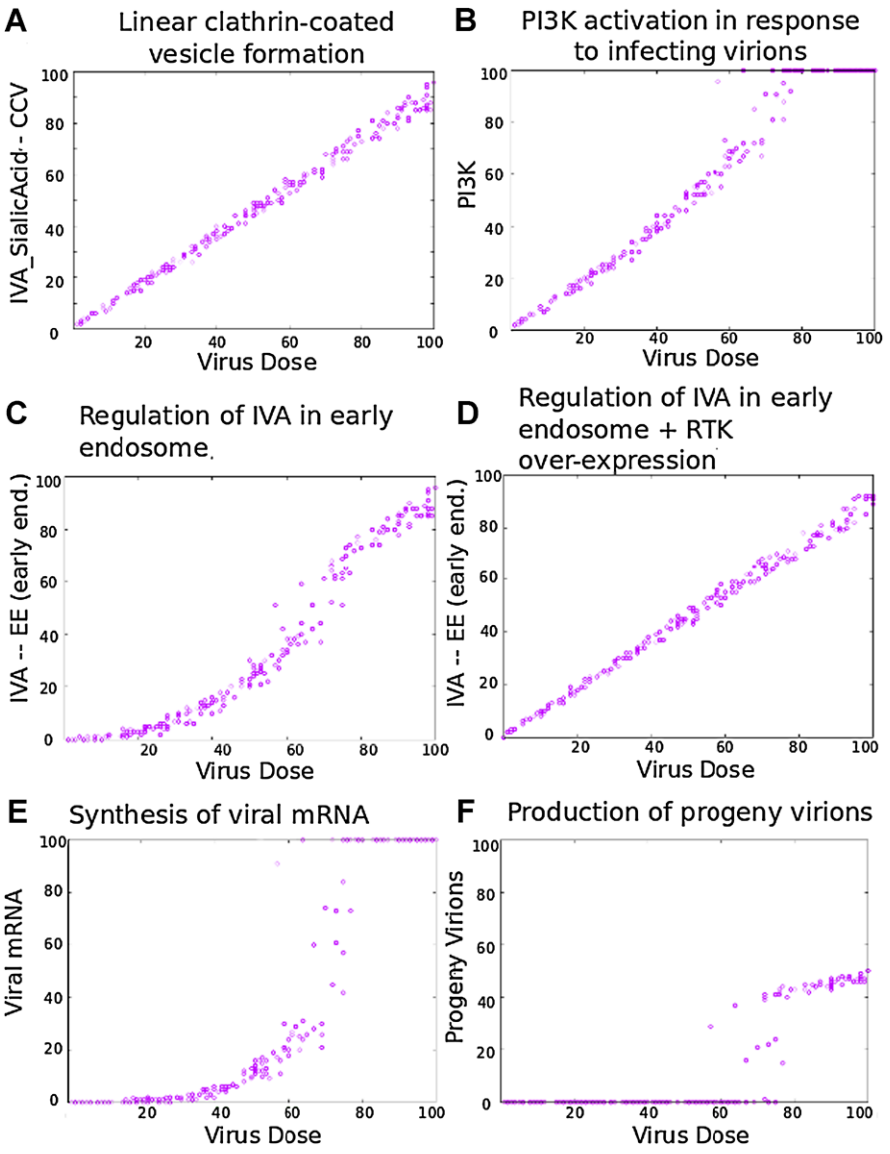

Fig. 3 Wild-type dynamics of influenza infection and replication. HA-sialic acid receptor affinity is assumed to be high (i.e., set to range randomly from 90–100 %). (**A**) Stimulation of sialic acid-virus formation in clathrin-coated vesicles by an increasing level of infecting virions. (**B**) The binding of infecting virions to the sialic receptor activates the RTK receptor-mediated PI3K pathway. (**C**) A delay effect of the feedforward loop integrated in the infection process. (**D**) Over-expression of RTK receptors leads to a more efficient cell entry of the virus. (**E**) Delayed & bistable synthesis of viral mRNA. (**F**) Bistable production of progeny virions

particles. The stimulation of the virus-receptor complex in early endosome is delayed due to the aforementioned feedforward loop (Fig. 3C). Recent laboratory studies also showed that the over-expression of the Epidermal Growth Factor Receptor (EGFR) results in an enhanced uptake of the virus in a host cell (Eierhoff et al. 2010). To further explore how the dynamics of the internalization of the virus is affected when the RTKs are over-expressed, we simulated the model in The Cell Collective with a "mutation" which renders the RTK node constitutively active. As shown in Panel D of Fig. 3 the delayed response is no longer present rendering the virus uptake a more efficient/responsive process. This is due to the fact that the RTK/PI3K side of the feedforward loop is now fully active as a result of RTK over-expression rendering the virus internalization solely dependent on the amount of infecting virions.

In investigating the dynamics of PI3K in Fig. 3B further we see that once the amount of infecting virions (Virus Dose) reaches a certain threshold (around 70 %) PI3K becomes fully active (and no longer responds to a further increase of infecting virions). This dynamic is a result of another architectural feature playing an important role in the regulatory network of influenza infection and replication cycle—the positive feedback loop.

Positive feedback loops are an integral part of many biological processes including signal transduction and gene regulation in mammalian cells (Bagowski and Ferrell 2001; Bagowski et al. 2003; Siciliano et al. 2011). The significance of positive feedback loops lies in the dynamics that arises from their structure. Specifically, positive feedback loops can lead to signal amplification or bistable behavior (Brandman and Meyer 2008). Bistability is a dynamical property of a system in which, once a certain signal threshold has been passed, it can move from an inactive to an active state. This bistable behavior can provide a basis for additional dynamical features; for example, in the budding yeast cell cycle a positive feedback loop gives rise to bistability which has been suggested to further result in an irreversible commitment of the cell to a round of division (Ferrell 2011).

In terms of our model, once the virus undergoes cellular internalization and subsequent translocation to the nucleus, replication begins. Figure 1 illustrates two main components of the influenza replication cycle. First, mRNA transcribed by the vRNP of the infecting virus (parental vRNP) is exported and translated into viral proteins (e.g., PB1, PA, NP, etc.). The second component (secondary transcription) is the synthesis of cRNA from the parental vRNP. The newly synthesized cRNA is then used to generate progeny vRNA (which eventually leads to the production of progeny virions), as well as to form new cRNP complexes which are in turn converted (indirectly) to additional vRNPs. Hence the production of progeny virions and its particles form a positive feedback loop. The effects of this positive feedback loop on the dynamics of the modeled viral replication cycle are twofold. First, as the viral protein NS1 is synthesized from newly produced viral mRNA during the replication cycle, it activates PI3K (Ehrhardt et al. 2007); because the new NS1 proteins are products of the positive feedback loop, the activation of PI3K is amplified once the positive feedback loop is activated later in the infection. This amplification effect is also seen in the dynamics of mRNA production (which is a precursor to NS1 production) in Fig. 3E. In fact, in the case of the mRNA, we can see both the delay effect of the feedforward loop as well as the amplification property of the positive feedback loop. Furthermore,

we were interested in the dynamical effects of the positive feedback loop on the levels of progeny virions produced. Simulations of our model suggest that the production of progeny viruses is bistable (Fig. 3F). That is, once an infection threshold is reached, the replication feedback loop is established, and progeny virions are produced.

Note that the "activation level" of progeny virions does not reach 100 %; this is due to the way we chose to model the process of exporting newly produced virus particles from the nucleus to the cytoplasm of the host cell—when the molecule in cytoplasm (e.g., mRNA_cyto) is activated by the same molecule type in the nucleus (e.g., mRNA_nuc), the molecule localized in the nucleus is consequently inactivated, indicating that it is leaving one location and entering a different one. Hence, the maximum activation level for mRNA in each nucleus and cytoplasm can be 50 %. This also re-iterates the fact that the activation levels do not represent specific biochemical enumerations (e.g., concentration, number of molecules, etc.); rather, it serves as a semi-quantitative/qualitative way to observe effects of various conditions on the dynamics of the system. Specifically, in the following sections, we present a number of *in silico* studies to demonstrate how our model is able to reproduce published laboratory experiments. These examples serve not only as a verification of our model but also as an instructional component to demonstrate how computational (and qualitative) models and computer simulations can be done to perform "dry" experiments.

4.3 The Role of PI3K in the Dynamics of Influenza Replication

In the previous section, simulations and analyses of the feedforward loop under normal conditions as well as during RTK over-expression suggest the importance of the RTK/PI3K pathway in response to the uptake of the virus (Eierhoff et al. 2010). In this section we further explore the role of PI3K interactions with the virus during infection. We use the model to follow the concepts used in a laboratory study done in Ehrhardt et al. (2006). As can be seen in the results of the dynamical simulations summarized in Fig. 4, the model is able to (qualitatively) reproduce many of the results from the aforementioned published paper. Specifically, Panels A–C in Fig. 4 show the inhibition of PI3K with wortmannin, an PI3K inhibitor, prevents the virus from reaching late endosome. Evidence also showed that treatment of wortmannin during the onset of infection reduced the level of progeny virions. Figure 4D–F shows that inhibiting PI3K results in the reduction of produced progeny virions. Because the external nodes (i.e., inhibitors and infecting virus particles) are activated simultaneously at the onset of the simulation (i.e., infection), Panels D–F correspond to the scenario when the inhibitor is added early in the infection. Hence, our simulation results agree with the experimental data. We also used the real-time simulation tool in The Cell Collective to investigate whether the addition of wortmannin later in the infection has any effects on the production of progeny virions. In fact, we found that once the production of progeny virions reaches its maximal level, the inhibition of PI3K has no effect (data not shown); presumably this is due to the fact that once the replication positive feedback loop is established, the inhibition of PI3K is unlikely to interrupt the virion production. Hence in the context of our model, inhibition of PI3K early could be an effective means to weaken virus infection.

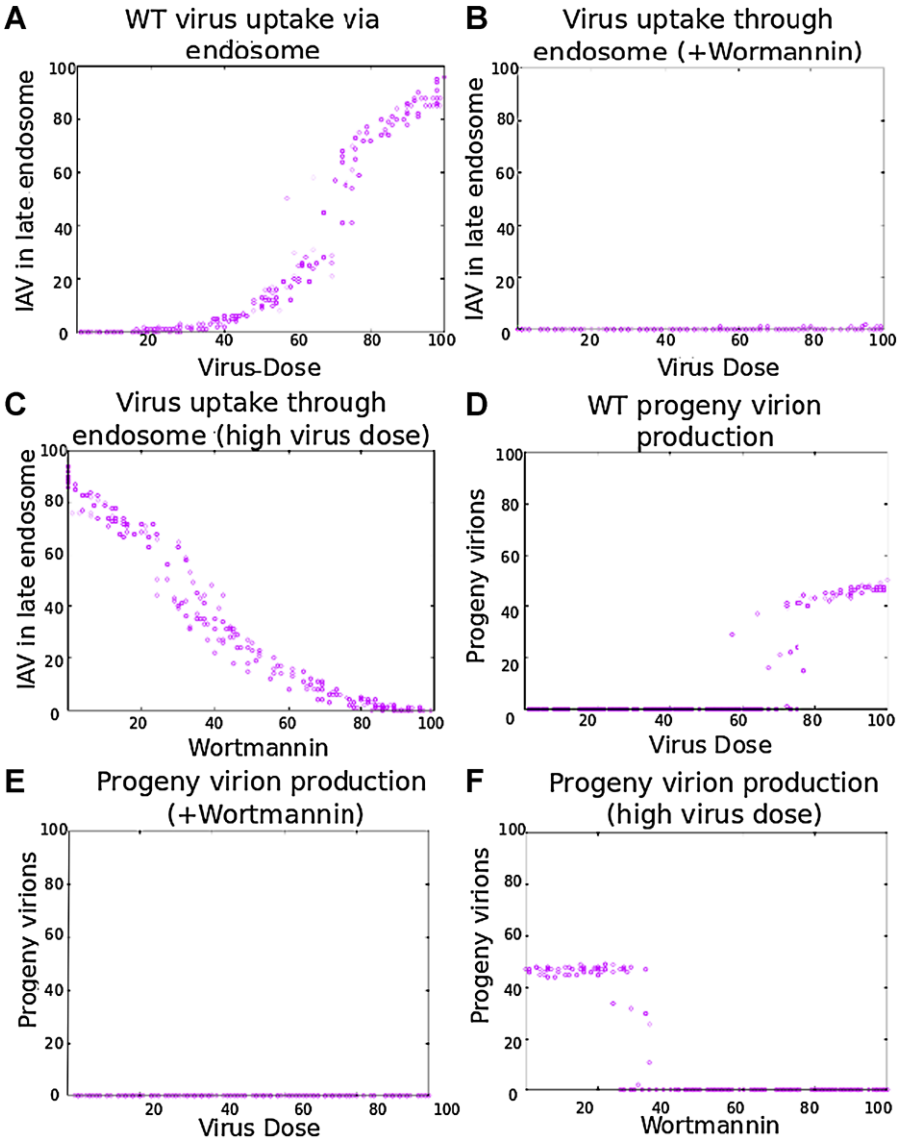

Fig. 4 PI3K is critical for efficient entry of influenza into the host cell. HA-sialic acid receptor affinity is assumed to be high (i.e., set to range randomly from 90–100 %). (**A**) Wild-type dynamics of virus endosytosis. (**B**) High levels of Wortmannin prevent the virus from reaching late endosome. (**C**) Increasing levels of Wortmannin prevent the virus from reaching late endosome. (**D**) Wild-type progeny virion production. (**E**) Inhibition of PI3K early in the infection prevents the initiation of the replication cycle, and hence production of progeny virions. (**F**) Increasing levels of Wortmannin reduce production of progeny virions

4.4 Dynamics of the Influenza-Raf/Mek/Erk Pathway Interactions

The Raf/Mek/Erk pathway governs a number of cellular functions such as growth, proliferation, and apoptosis (Daum et al. 1994; Matallanas et al. 2011). This pathway

plays an important role in a number of diseases, including cancer. It has also been found that Erk-NS2 interaction is essential for proper export of vRNPs from nucleus to the cytoplasm and overall replication of the virus (Pleschka et al. 2001). In our model, we included the Raf/Mek/Erk pathway (see Fig. 2) activated by multivalent binding and raft signaling domain formation and its interaction with NS2. To simulate the effects of Mek inhibition as done in the aforementioned laboratory experiment, an external input species representing the U0126 Mek inhibitor was also added. Figure 5 illustrates the dynamics of progeny export from the nucleus to cytoplasm under wild-type conditions (Pleschka et al. 2001) (Panels A & B). Addition of high levels of the Mek inhibitor (U0126) results in the retention of progeny vRNP in the nucleus and minimized export to the cytoplasm, Panels C and D, respectively. Panels E and F illustrate the dose-response relationship between the inhibitor and the retention or export of progeny vRNP from the nucleus to the cytoplasm.

4.5 PKC Inhibition Prevents Viral Entry into the Nucleus

Similar to the Erk pathway discussed above, protein kinase C (PKC) also plays a role in influenza infection (Root et al. 2000). Cells untreated with PKC inhibitor Bisindolylmaleimide I contained NA on the plasma membrane and NP in the cytoplasm whereas treated cells did not have NA or NP expression in a dose-dependent manner (Fig. 3 in Root et al. 2000), suggesting replication inhibition by preventing some point before vRNP entry into nucleus. Depending on Bisindolylmaleimide I levels, NA and NP expression was suggested to have an 'all-or-nothing' effect. To reproduce the immunofluorescence studies on NP and NA, an external negative regulator was added to our model representing the PKC inhibitor Bisindolylmaleimide I (Fig. 6). Comparing Panels A and D with B and E (Fig. 6) demonstrate the laboratory observation of an 'all-or-nothing' effect of low (0–30 %) and high (90–100 %) levels of Bisindolylmaleimide I. Also consistent with laboratory observations is the dose-dependent inhibition of NP and NA localization with varying Bisindolylmaleimide I levels (Panels C and F).

It was determined by Sieczkarski et al. (2003) that PKC is a regulator of proper late endosome formation and function. Cells lacking PKC activity result in influenza virus accumulation in cytoplasmic vesicles. Consistent with these findings, Panels G and H show relatively similar early endosome maturation levels with wild type (no inhibitors) and high Bisindolylmaleimide I, suggesting no role of PKC in early endosome maturation (Panel I shows little/no correlation between early endosome maturation with varying levels of Bisindolylmaleimide I). However, wild-type late endosome formation (Panel J) is strikingly different from that of with high Bisindolylmaleimide levels (Panel K) suggesting the requirement of PKC for late endosome formation. The dose response of late endosome formation to increasing Bisindolylmaleimide I is shown in panel L.

4.6 Comparing and Predicting Drug Effectiveness

The 2009 H1N1 "swine flu" recently demonstrated problems with our current vaccination programs—inadequate pace in manufacture and distribution in comparison

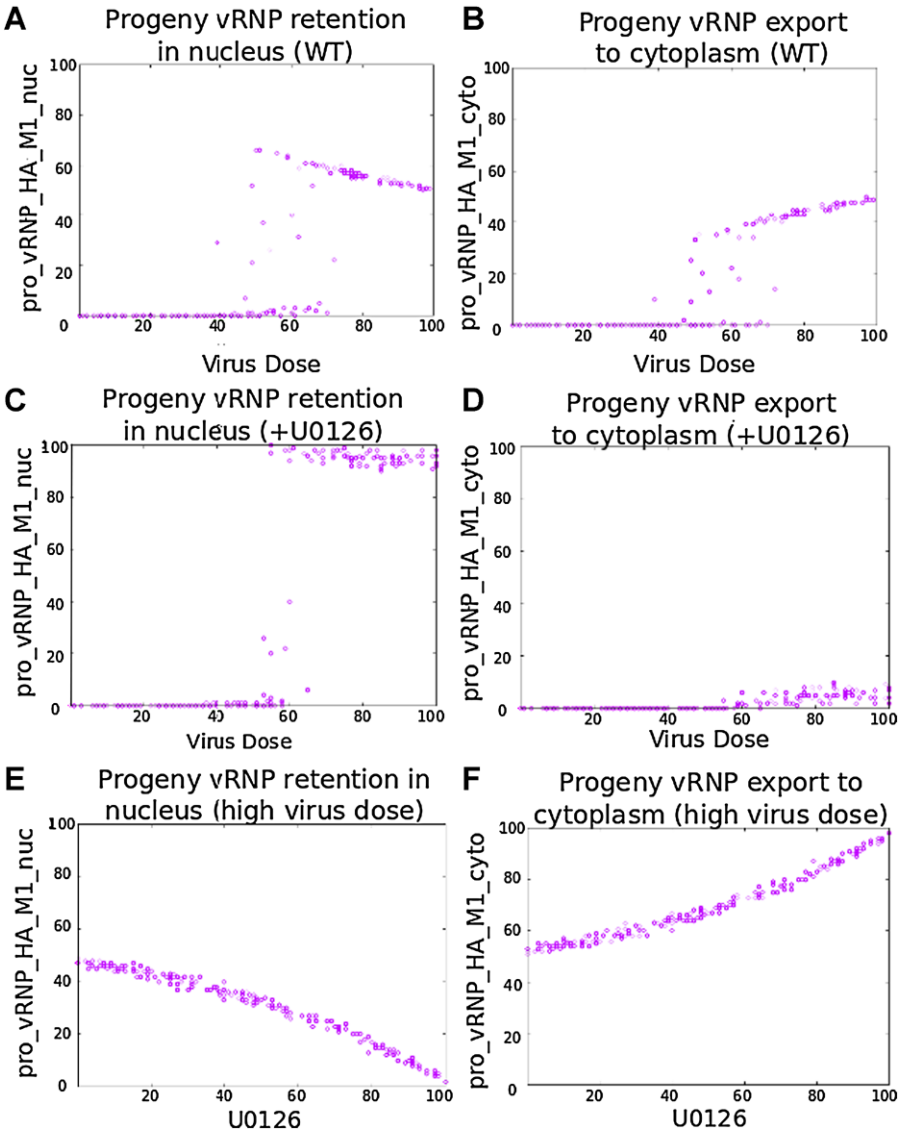

Fig. 5 Mek plays a key role in the export of progeny vRNP from nucleus to cytoplasm. HA-sialic acid receptor affinity is assumed to be high (i.e., set to range randomly from 90–100 %). (**A**) Accumulation of progeny vRNP in nucleus under wild-type conditions. (**B**) Export of progeny vRNP from nucleus to cytoplasm under wild-type conditions. (**C**) Maximized accumulation of progeny vRNP in nucleus with high levels of U0126 inhibitor. (**D**) Minimized export of progeny vRNP from nucleus to cytoplasm with high levels of U0126. (**E**) Increasing levels of U0126 result in decreased retention of progeny vRNP in the nucleus. (**F**) Increasing levels of U0126 result in increased export of progeny vRNP to cytoplasm

to a rapidly spreading pandemic virus (Broadbent and Subbarao 2011). Thus in the early phase of a pandemic, the best way to fight influenza is through antiviral drugs. Currently approved drugs target viral proteins such as NA (Zanamivir/Oseltamivir)

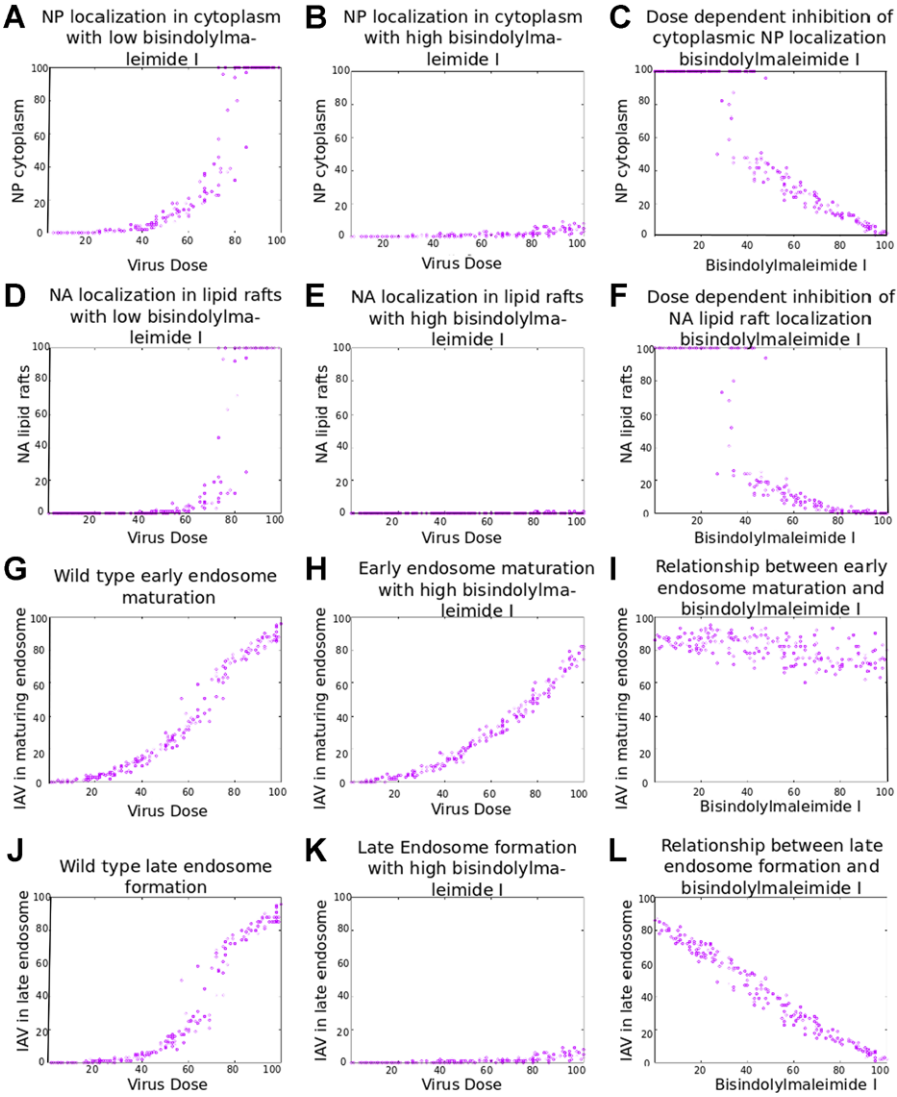

Fig. 6 PKC is important in the process of viral entry into the nucleus. HA-sialic acid receptor affinity is assumed to be high (i.e., set to range randomly from 90–100 %). (**A**) NP in cytoplasm as a result of increasing levels of infecting virus particles; Low amount (0–30 %) of PKC inhibitor was added. (**B**) Same as **A**—with high levels (90–100 %) of PKC inhibitor. (**C**) Increasing levels of the PKC inhibitor result in decreased localization of NP. (**D**–**F**) Same as **A**–**C** while measuring NA localization in lipid rafts. (**G**) Early endosome maturation under WT conditions (i.e., no inhibitors). (**H**) Effects of high level of Bisindolylmaleimide I on endosome maturation. (**I**) Dose response of endosome maturation to increasing levels of Bisindolylmaleimide I. (**J**) Late endosome formation under WT conditions. (**K**) High levels of Bisindolylmaleimide I significantly reduce formation of late endosome. (**L**) Increasing levels of Bisindolylmaleimide I result in decreased formation of late endosome

and an M2 ion channel protein (Amantadine/Rimantadine) (De Clercq 2006). Unfortunately surface proteins, such as NA and M2, are subject to high antigenic mutation rates resulting in an accelerated drug resistance. Amantadine/Rimantadine is not effective against currently circulating A (H3N2) and 2009 pandemic influenza A (H1N1) viruses. Emergence of strains resistant to NA inhibitors Zanamivir/Oseltamivir is becoming more prevalent, demonstrating the need for broader antiviral options (Fry and Gubareva 2012; Ison 2011).

To overcome drug resistance, identifying target viral proteins with less likelihood of mutations is becoming more appealing (Gillis 2006; Nakazawa et al. 2008; Wilkinson et al. 2012). For instance, heterotrimeric polymerase (3P), vital for viral transcription and replication, was identified as a target in that the inhibition with a potent RNA polymerase inhibitor L-741,001 resulted in complete inhibition of virus replication (Nakazawa et al. 2008). Targeting of the polymerase subunit proved to be more effective than NA inhibitor Zanamivir for virus re-growth (Nakazawa et al. 2008). However, while the use of L-741.001 had much lower resistance than NA, gradual and long-term mutations can render the drug obsolete.

Targeting of the PB1:PA interface may be more promising. PB1 is central for 3P formation as it contains two domains that interact with the PB2 and PA subunits. The N-terminal and the C-terminal regions of PB1 bind to PA and PB2 subunits, respectively (Biswas and Nayak 1996; González et al. 1996; Ohtsu et al. 2002; Pérez and Donis 1995; Poole et al. 2007). Sequence analysis has shown that the PA-binding domain of PB1 is highly conserved as multiple offsetting mutations in both PA and PB1 would be required. Similar to L-741,001, the use of binding peptides at the PB1:PA interface blocked viral polymerase activity and inhibited viral replication (Ghanem et al. 2007; Wunderlich et al. 2009). This interface has thus been proposed to be druggable, and a 25-residue PB1-derived peptide has been shown to inhibit polymerase assembly and virus replication (Boivin et al. 2010).

To predict and compare the effectiveness of the proposed PB1:PA target against currently approved drugs (Amantadine/Rimantadine and Zanamivir/Oseltamivir), we simulated the presented model under increasing doses of each drug. Results in Fig. 7 suggest that while all drugs lead to complete reduction of progeny virions, a drug proposed to target PB1:PA is the most effective in reducing the production of progeny virions (in the context of the model). Upon investigating the differences in the infection dynamics as a response to the aforementioned drugs, these results point in the direction of the most appropriate place of intervention during influenza infection. Specifically, the all-or-none response to Amantadine/Rimantadine after a threshold is reached (Fig. 7A) appears to be due to the fact that this drug targets M2 in an early step of the influenza life cycle by retaining incoming vRNPs in endosomal compartments for which the protein M2 is required (Pinto et al. 1992; Takeda et al. 2002). Hence, unless the virus is sufficiently prevented from entering the nucleus of the host cell, the replicative positive feedback loop will be initiated resulting in the production of an unaffected amount of progeny virions.

Figure 7B shows gradual, yet more efficient than Amantadin/Rimantaine treatment, decrease in progeny virions in the presence of the NA inhibitor Zanamivir/Oseltamivir. The observed (linear) dynamic corresponds to the fact NA is critical in the last step of the influenza life cycle—budding of the progeny virions and its release from infected cells (Gubareva et al. 2000; Gong et al. 2007;

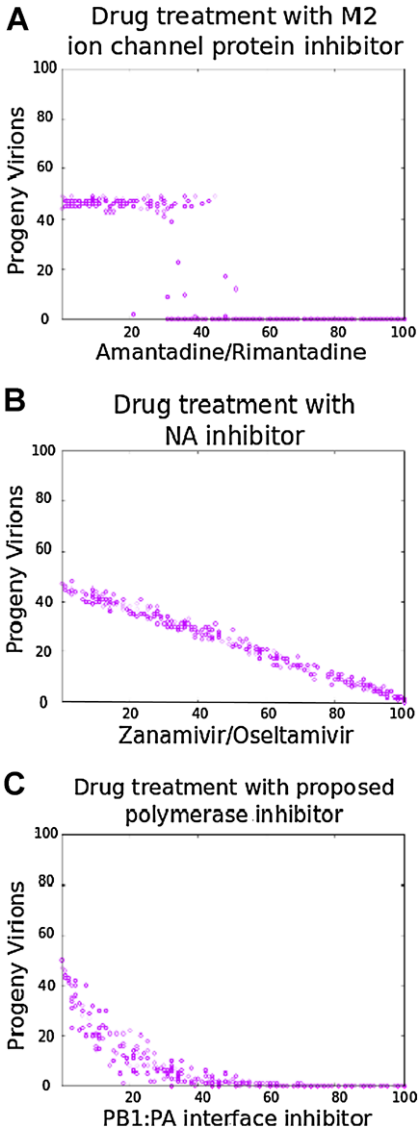

Fig. 7 Potential drug targeting the PB1:PA interface is predicted to be more effective than Amantadine/Rimantadine or Zanamivir/Pseltamivir. HA-sialic acid receptor affinity and viral dose are assumed to be high (i.e., set to range randomly from 90–100 %). (**A**) All-or-none response of the production of progeny virions to increasing amount of Amantadine/Rimantadine. (**B**) Targeting NA (i.e., progeny virion exit) is more effective than targeting the M2 ion channel. (**C**) The PB1:PA interface is predicted to be the most effective drug target

Skehel 2009). Finally, simulation results in Fig. 7C indicate that the intervention of the PB1:PA complex is the most efficient of the three compared targets. From the dynamics perspective, this efficiency is likely to be due to the fact that this interaction lies the center of the replication cycle affecting the replicative positive feedback loop. (In future studies, we plan to examine all critical components contained in this loop.) In addition, PB1:PA has very well conserved binding domains that are resistant to antigenic drift (Ghanem et al. 2007) hence providing a promising target for future antiviral therapy development.

5 Discussion and Conclusions

Herein we present the first comprehensive dynamical model of a regulatory network governing the process of influenza infection and replication. Using The Cell Collective, a modeling platform that allows scientists to conduct virtual experiments similar to the way they are performed in the laboratory, we explored the dynamics of the influenza model under wild-type conditions as well as under the use of inhibitors targeting a number of host pathways important in the infection and replication of influenza. Also demonstrated was the importance and dynamics of a feedforward loop identified to regulate the cell entry stage of the infection, as well as a positive feedback loop, governing the replication cycle of the virus and production of progeny virions. Our model was able to reproduce the qualitative behavior of the molecular dynamics observed experimentally. Finally, we used the model to compare and predict the effectiveness of Zanamivir/Oseltamivir and Amantadine/Rimantadine, and a proposed inhibitor of PB1:PA. Our findings suggest that a drug developed to target the PB1:PA complex would be more effective than the compared, existing drugs.

Despite the correctly reproduced behavior, the model is still limited in that many of the modeled host interactions are relatively simple; that is, it is well established that the host biochemical pathways such as PI3K, Erk, PKC, etc., as well as others currently completely omitted from the model (e.g., cytokine pathways) are a part of a much more complex biochemical network. Hence, it will be important, in future studies, to expand this model and include additional components that may prove to be a potential target for anti-influenza therapy. Though we are currently working on one of the largest virtual epithelial cells, which will form a basis for the integration of the model with the presented influenza system, qualitative modeling opens doors for laboratory scientists to engage in expanding these computational models.

This modeling is now possible with The Cell Collective platform designed to enable community-based model building. Because of the inherent size and complexity of biochemical networks, it is extremely difficult for a single person or group to efficiently transfer the vast amount of laboratory data into a mathematical representation; this fact applies to any modeling technique. One way to address this issue is to engage the community of laboratory scientists that have generated these data and, hence, have first-hand knowledge of the local protein-protein regulatory mechanisms. The Cell Collective provides a mechanism by which they can collaborate by contributing their intimate knowledge of local interactions into a large-scale global model, which can be greatly enhanced in terms of both size and accuracy. Since parameter-independent models rely mostly on qualitative information, it makes them an ideal candidate for such a platform as most laboratory scientists communicate their data in qualitative terms. Thus, it can be argued that the key to relatively complete large-scale dynamical models and virtual cells is the global engagement of laboratory scientists with "biologist-friendly" software tools that bridge the gap between the mathematical modeling and the general scientific community.

References

Abdi, A., Tahoori, M. B., & Emamian, E. S. (2008). Fault diagnosis engineering of digital circuits can identify vulnerable molecules in complex cellular pathways. *Sci. Signal.*, *1*(42), ra10.

Alon, U. (2007). Network motifs: theory and experimental approaches. *Nat. Rev. Genet.*, *8*(6), 450–461.
Bagowski, C. P., & Ferrell, J. E. (2001). Bistability in the JNK cascade. *Curr. Biol., CB*, *11*(15), 1176–1182.
Bagowski, C. P., Besser, J., Frey, C. R., & Ferrell, J. E. (2003). The JNK cascade as a biochemical switch in mammalian cells: ultrasensitive and all-or-none responses. *Curr. Biol., CB*, *13*(4), 315–320.
Beauchemin, C., Samuel, J., & Tuszynski, J. (2005). A simple cellular automaton model for influenza A viral infections. *J. Theor. Biol.*, *232*(2), 223–234.
Beyer, T., Busse, M., Hristov, K., Gurbiel, S., Smida, M., Haus, U.-U., Ballerstein, K., Pfeuffer, F., Weismantel, R., Schraven, B., & Lindquist, J. A. (2011). Integrating signals from the T-cell receptor and the interleukin-2 receptor. *PLoS Comput. Biol.*, *7*(8), e1002121.
Biswas, S. K., & Nayak, D. P. (1996). Influenza virus polymerase basic protein 1 interacts with influenza virus polymerase basic protein 2 at multiple sites. *J. Virol.*, *70*(10), 6716–6722.
Boivin, S., Cusack, S., Ruigrok, R. W. H., & Hart, D. J. (2010). Influenza A virus polymerase: structural insights into replication and host adaptation mechanisms. *J. Biol. Chem.*, *285*(37), 28411–28417.
Boulo, S., Akarsu, H., Ruigrok, R. W. H., & Baudin, F. (2007). Nuclear traffic of influenza virus proteins and ribonucleoprotein complexes. *Virus Res.*, *124*(1–2), 12–21.
Boyer, L. A., Lee, T. I., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., Guenther, M. G., Kumar, R. M., Murray, H. L., Jenner, R. G., Gifford, D. K., Melton, D. A., Jaenisch, R., & Young, R. A. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell*, *122*(6), 947–956.
Brandman, O., & Meyer, T. (2008). Feedback loops shape cellular signals in space and time. *Science (New York, NY)*, *322*(5900), 390–395.
Broadbent, A. J., & Subbarao, K. (2011). Influenza virus vaccines: lessons from the 2009 H1N1 pandemic. *Curr. Opin. Virol.*, *1*(4), 254–262.
Chaves, M., Albert, R., & Sontag, E. D. (2005). Robustness and fragility of Boolean models for genetic regulatory networks. *J. Theor. Biol.*, *235*(3), 431–449.
Chaves, M., Sontag, E. D., & Albert, R. (2006). Methods of robustness analysis for Boolean models of gene control networks. *Syst. Biol.*, *153*(4), 154–167.
Cheng, D., & Qi, H. (2010). State-space analysis of Boolean networks. *IEEE Trans. Neural Netw.*, *21*(4), 584–594. A publication of the IEEE Neural Networks Council
Daum, G., Eisenmann-Tappe, I., Fries, H. W., Troppmair, J., & Rapp, U. R. (1994). The ins and outs of Raf kinases. *Trends Biochem. Sci.*, *19*(11), 474–480.
De Clercq, E. (2006). Antiviral agents active against influenza A viruses. *Nat. Rev. Drug Discov.*, *5*(12), 1015–1025.
Detjen, B. M., St Angelo, C., Katze, M. G., & Krug, R. M. (1987). The three influenza virus polymerase (P) proteins not associated with viral nucleocapsids in the infected cell are in the form of a complex. *J. Virol.*, *61*(1), 16–22.
Ehrhardt, C., Marjuki, H., Wolff, T., Nürnberg, B., Planz, O., Pleschka, S., & Ludwig, S. (2006). Bivalent role of the phosphatidylinositol-3-kinase (PI3K) during influenza virus infection and host cell defence. *Cell. Microbiol.*, *8*(8), 1336–1348.
Ehrhardt, C., Wolff, T., Pleschka, S., Planz, O., Beermann, W., Bode, J. G., Schmolke, M., & Ludwig, S. (2007). Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. *J. Virol.*, *81*(7), 3058–3067.
Eierhoff, T., Hrincius, E. R., Rescher, U., Ludwig, S., & Ehrhardt, C. (2010). The epidermal growth factor receptor (EGFR) promotes uptake of influenza A viruses (IAV) into host cells. *PLoS Pathog.*, *6*(9), e1001099.
Emmert-Streib, F., & Dehmer, M. (2009). Information processing in the transcriptional regulatory network of yeast: functional robustness. *BMC Syst. Biol.*, *3*(35).
Engelhardt, O. G., & Fodor, E. (2006). Functional association between viral and cellular transcription during influenza virus infection. *Reviews in medical virology*, *16*(5), 329–345.
Ferrell, J. E. (2011). Simple rules for complex processes: new lessons from the budding yeast cell cycle. *Mol. Cell*, *43*(4), 497–500.
Forst, C. V. (2006). Host-pathogen systems biology. *Drug Discov. Today*, *11*(5–6), 220–227.
Fry, A. M., & Gubareva, L. V. (2012). Understanding influenza virus resistance to antiviral agents; early warning signs for wider community circulation. *J. Inf. Dis.*
Ghanem, A., Mayer, D., Chase, G., Tegge, W., Frank, R., Kochs, G., García-Sastre, A., & Schwemmle, M. (2007). Peptide-mediated interference with influenza A virus polymerase. *J. Virol.*, *81*(14), 7801–7804.

Gillis, J. S. (2006). An avian influenza vaccine for humans targeting the polymerase B2 protein inside the capsid instead of hemagglutinin or neuramidase on the virus surface. *Med. Hypotheses*, *66*(5), 975–977.

Gong, J., Xu, W., & Zhang, J. (2007). Structure and functions of influenza virus neuraminidase. *Curr. Med. Chem.*, *14*(1), 113–122.

González, S., Zürcher, T., & Ortín, J. (1996). Identification of two separate domains in the influenza virus PB1 protein involved in the interaction with the PB2 and PA subunits: a model for the viral RNA polymerase structure. *Nucleic Acids Res.*, *24*(22), 4456–4463.

Gubareva, L. V., Kaiser, L., & Hayden, F. G. (2000). Influenza virus neuraminidase inhibitors. *Lancet*, *355*(9206), 827–835.

Hancioglu, B., Swigon, D., & Clermont, G. (2007). A dynamical model of human immune response to influenza A virus infection. *J. Theor. Biol.*, *246*(1), 70–86.

Helikar, T., & Rogers, J. A. (2009). ChemChains: a platform for simulation and analysis of biochemical networks aimed to laboratory scientists. *BMC Syst. Biol.*, *3*, 58.

Helikar, T., Konvalina, J., Heidel, J., & Rogers, J. A. (2008). Emergent decision-making in biological signal transduction networks. *Proc. Natl. Acad. Sci. USA*, *105*(6), 1913–1918.

Helikar, T. T., Kochi, N., Konvalina, J., & Rogers, J. A. (2011). Boolean modeling of biochemical networks. *Open Bioinforma. J.*, *4*(5), 16–25.

Helikar, T., Kowal, B., McClenathan, S., Bruckner, M., Rowley, T., Madrahimov, A., Wicks, B., Shrestha, M., Limbu, K., & Rogers, J. A. (2012a). The cell collective: toward an open and collaborative approach to systems biology. *BMC Syst. Biol.*, *6*(1), 96.

Helikar, T., Kowal, B., Madrahimov, A., Shrestha, M., Pedersen, J., Konvalina, J., & Rogers, J. A. (2012b). Bio-Logic Builder: a non-technical tool for building dynamical, qualitative models. *PLoS ONE*, *7*(10), e46417.

Herz, C., Stavnezer, E., Krug, R., & Gurney, T. (1981). Influenza virus, an RNA virus, synthesizes its messenger RNA in the nucleus of infected cells. *Cell*, *26*(3 Pt 1), 391–400.

Ison, M. G. (2011). Antivirals and resistance: influenza virus. *Curr. Opin. Virol.*, *1*(6), 563–573.

Kitano, H. (2002). Systems biology: a brief overview. *Science*, *295*(5560), 1662–1664.

König, R., Stertz, S., Zhou, Y., Inoue, A., Hoffmann, H.-H., Bhattacharyya, S., Alamares, J. G., Tscherne, D. M., Ortigoza, M. B., Liang, Y., Gao, Q., Andrews, S. E., Bandyopadhyay, S., De Jesus, P., Tu, B. P., Pache, L., Shih, C., Orth, A., Bonamy, G., Miraglia, L., Ideker, T., García-Sastre, A., Young, J. A. T., Palese, P., Shaw, M. L., & Chanda, S. K. (2010). Human host factors required for influenza virus replication. *Nature*, *463*(7282), 813–817.

Krumbholz, A., Philipps, A., Oehring, H., Schwarzer, K., Eitner, A., Wutzler, P., & Zell, R. (2011). Current knowledge on PB1-F2 of influenza A viruses. *Med. Microbiol. Immunol.*, *200*(2), 69–75.

Lee, T. I., Rinaldi, N. J., Robert, F., Odom, D. T., Bar-Joseph, Z., Gerber, G. K., Hannett, N. M., Harbison, C. T., Thompson, C. M., Simon, I., Zeitlinger, J., Jennings, E. G., Murray, H. L., Gordon, D. B., Ren, B., Wyrick, J. J., Tagne, J.-B., Volkert, T. L., Fraenkel, E., Gifford, D. K., & Young, R. A. (2002). Transcriptional regulatory networks in saccharomyces cerevisiae. *Science (New York, NY)*, *298*(5594), 799–804.

Ludwig, S., Pleschka, S., Planz, O., & Wolff, T. (2006). Ringing the alarm bells: signalling and apoptosis in influenza virus infected cells. *Cell. Microbiol.*, *8*(3), 375–386.

Ma'ayan, A., Jenkins, S. L., Neves, S., Hasseldine, A., Grace, E., Dubin-Thaler, B., Eungdamrong, N. J., Weng, G., Ram, P. T., Rice, J. J., Kershenbaum, A., Stolovitzky, G. A., Blitzer, R. D., & Iyengar, R. (2005). Formation of regulatory patterns during signal propagation in a Mammalian cellular network. *Science (New York, NY)*, *309*(5737), 1078–1083.

Mangan, S., & Alon, U. (2003). Structure and function of the feed-forward loop network motif. *Proc. Natl. Acad. Sci. USA*, *100*(21), 11980–11985.

Mangan, S., Zaslaver, A., & Alon, U. (2003). The coherent feedforward loop serves as a sign-sensitive delay element in transcription networks. *J. Mol. Biol.*, *334*(2), 197–204.

Matallanas, D., Birtwistle, M., Romano, D., Zebisch, A., Rauch, J., von Kriegsheim, A., & Kolch, W. (2011). Raf family kinases: old dogs have learned new tricks. *Genes Cancer*, *2*(3), 232–260.

Milo, R., Shen-Orr, S., Itzkovitz, S., Kashtan, N., Chklovskii, D., & Alon, U. (2002). Network motifs: simple building blocks of complex networks. *Science (New York, NY)*, *298*(5594), 824–827.

Morris, M. K., Saez-Rodriguez, J., Sorger, P. K., & Lauffenburger, D. A. (2010). Logic-based models for the analysis of cell signaling networks. *Biochemistry*, *49*(15), 3216–3224.

Nakazawa, M., Kadowaki, S., Watanabe, I., Kadowaki, Y., Takei, M., & Fukuda, H. (2008). PA subunit of RNA polymerase as a promising target for anti-influenza virus agents. *Antivir. Res.*, *78*(3), 194–201.

Naldi, A., Carneiro, J., Chaouiya, C., & Thieffry, D. (2010). Diversity and plasticity of Th cell types predicted from regulatory network modelling. *PLoS Comput. Biol.*, *6*(9), e1000912.

Odom, D. T., Zizlsperger, N., Gordon, D. B., Bell, G. W., Rinaldi, N. J., Murray, H. L., Volkert, T. L., Schreiber, J., Rolfe, P. A., Gifford, D. K., Fraenkel, E., Bell, G. I., & Young, R. A. (2004). Control of pancreas and liver gene expression by HNF transcription factors. *Science (New York, NY)*, *303*(5662), 1378–1381.

Ohtsu, Y., Honda, Y., Sakata, Y., Kato, H., & Toyoda, T. (2002). Fine mapping of the subunit binding sites of influenza virus RNA polymerase. *Microbiol. Immunol.*, *46*(3), 167–175.

O'Neill, R. E., Talon, J., & Palese, P. (1998). The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. *EMBO J.*, *17*(1), 288–296.

Oslund, K. L., & Baumgarth, N. (2011). Influenza-induced innate immunity: regulators of viral replication, respiratory tract pathology & adaptive immunity. *Future Virol.*, *6*(8), 951–962.

Payungporn, S., Panjaworayan, N., Makkoch, J., & Poovorawan, Y. (2010). Molecular characteristics of the human pandemic influenza A virus (H1N1). *Acta Virol.*, *54*(3), 155–163.

Pérez, D. R., & Donis, R. O. (1995). A 48-amino-acid region of influenza A virus PB1 protein is sufficient for complex formation with PA. *J. Virol.*, *69*(11), 6932–6939.

Pinto, L. H., Holsinger, L. J., & Lamb, R. A. (1992). Influenza virus M2 protein has ion channel activity. *Cell*, *69*(3), 517–528.

Pleschka, S., Wolff, T., Ehrhardt, C., Hobom, G., Planz, O., Rapp, U. R., & Ludwig, S. (2001). Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade. *Nat. Cell Biol.*, *3*(3), 301–305.

Poole, E. L., Medcalf, L., Elton, D., & Digard, P. (2007). Evidence that the C-terminal PB2-binding region of the influenza A virus PB1 protein is a discrete alpha-helical domain. *FEBS Lett.*, *581*(27), 5300–5306.

Rodríguez, A., Sosa, D., Torres, L., Molina, B., Frías, S., & Mendoza, L. (2012). A Boolean network model of the FA/BRCA pathway. *Bioinformatics (Oxford, England)*, *28*(6), 858–866.

Root, C. N., Wills, E. G., McNair, L. L., & Whittaker, G. R. (2000). Entry of influenza viruses into cells is inhibited by a highly specific protein kinase C inhibitor. *J. Gen. Virol.*, *81*(Pt 11), 2697–2705.

Rossman, J. S., & Lamb, R. A. (2011). Influenza virus assembly and budding. *Virology*, *411*(2), 229–236.

Saez-Rodriguez, J., Simeoni, L., Lindquist, J. A., Hemenway, R., Bommhardt, U., Arndt, B., Haus, U.-U., Weismantel, R., Gilles, E. D., Klamt, S., & Schraven, B. (2007). A logical model provides insights into T cell receptor signaling. *PLoS Comput. Biol.*, *3*(8), e163.

Shapira, S. D., Gat-Viks, I., Shum, B. O. V., Dricot, A., de Grace, M. M., Wu, L., Gupta, P. B., Hao, T., Silver, S. J., Root, D. E., Hill, D. E., Regev, A., & Hacohen, N. (2009). A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. *Cell*, *139*(7), 1255–1267.

Shen-Orr, S. S., Milo, R., Mangan, S., & Alon, U. (2002). Network motifs in the transcriptional regulation network of Escherichia coli. *Nat. Genet.*, *31*(1), 64–68.

Siciliano, V., Menolascina, F., Marucci, L., Fracassi, C., Garzilli, I., Moretti, M. N., & di Bernardo, D. (2011). Construction and modelling of an inducible positive feedback loop stably integrated in a mammalian cell-line. *PLoS Comput. Biol.*, *7*(6), e1002074.

Sidorenko, Y., & Reichl, U. (2004). Structured model of influenza virus replication in MDCK cells. *Biotechnol. Bioeng.*, *88*(1), 1–14.

Sieczkarski, S. B., Brown, H. A., & Whittaker, G. R. (2003). Role of protein kinase C betaII in influenza virus entry via late endosomes. *J. Virol.*, *77*(1), 460–469.

Skehel, J. (2009). An overview of influenza haemagglutinin and neuraminidase. *Biol., J. Int. Assoc. Biol. Stand.*, *37*(3), 177–178.

Song, B. M., Kang, Y. M., Kim, H. S., & Seo, S. H. (2011). Induction of inflammatory cytokines and toll-like receptors in human normal respiratory epithelial cells infected with seasonal H1N1, 2009 pandemic H1N1, seasonal H3N2, and highly pathogenic H5N1 influenza virus. *Viral Immunol.*, *24*(3), 179–187.

Takeda, M., Pekosz, A., Shuck, K., Pinto, L. H., & Lamb, R. A. (2002). Influenza a virus M2 ion channel activity is essential for efficient replication in tissue culture. *J. Virol.*, *76*(3), 1391–1399.

Trautmann, L., & Sekaly, R.-P. (2011). Solving vaccine mysteries: a systems biology perspective. *Nat. Immunol.*, *12*(8), 729–731.

Turan, K., Mibayashi, M., Sugiyama, K., Saito, S., Numajiri, A., & Nagata, K. (2004). Nuclear MxA proteins form a complex with influenza virus NP and inhibit the transcription of the engineered influenza virus genome. *Nucleic Acids Res.*, *32*(2), 643–652.

Umezawa, T. (2011). Systems biology approaches to abscisic acid signaling. *J. Plant Res.*, *124*(4), 539–548.

Watanabe, T., Watanabe, S., & Kawaoka, Y. (2010). Cellular networks involved in the influenza virus life cycle. *Cell Host Microbe*, *7*(6), 427–439.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., & Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol. Rev.*, *56*(1), 152–179.

Wilkinson, T. M., Li, C. K. F., Chui, C. S. C., Huang, A. K. Y., Perkins, M., Liebner, J. C., Lambkin-Williams, R., Gilbert, A., Oxford, J., Nicholas, B., Staples, K. J., Dong, T., Douek, D. C., McMichael, A. J., & Xu, X.-N. (2012). Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans. *Nat. Med.*, *18*(2), 274–280.

Wunderlich, K., Mayer, D., Ranadheera, C., Holler, A.-S., Mänz, B., Martin, A., Chase, G., Tegge, W., Frank, R., Kessler, U., & Schwemmle, M. (2009). Identification of a PA-binding peptide with inhibitory activity against influenza A and B virus replication. *PLoS ONE*, *4*(10), e7517.

Yan, Q. (2010). Systems biology of influenza: understanding multidimensional interactions for personalized prevention and treatment. *Methods Mol. Biol.*, *662*, 285–302.

Zheng, J., Zhang, D., Przytycki, P. F., Zielinski, R., Capala, J., & Przytycka, T. M. (2010). SimBoolNet—a cytoscape plugin for dynamic simulation of signaling networks. *Bioinformatics (Oxford, England)*, *26*(1), 141–142.