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Systems biology of virus-host signaling network interactions

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Viruses have evolved to manipulate the host cell machinery for virus propagation, in part by interfering with the host cellular signaling network. Molecular studies of individual pathways have uncovered many viral host-protein targets; however, it is difficult to predict how viral perturbations will affect the signaling network as a whole. Systems biology approaches rely on multivariate, context-dependent measurements and computational analysis to elucidate how viral infection alters host cell signaling at a network level. Here we describe recent advances in systems analyses of signaling networks in both viral and non-viral biological contexts. These approaches have the potential to uncover virus- mediated changes to host signaling networks, suggest new therapeutic strategies, and assess how cell-to-cell variability affects host responses to infection. We argue that systems approaches will both improve understanding of how individual virus-host protein interactions fit into the progression of viral pathogenesis and help to identify novel therapeutic targets. [BMB reports 2012; 45(4): 213-220]

INTRODUCTION

Cells respond to environmental changes by translating a complex network of protein interactions and biochemical signaling reactions into functional responses (e.g., cytokine secretion or cell death). In human diseases such as pathogenic viral infections and cancer, malfunctioning signaling networks cause cells to incorrectly respond to stimuli and produce the diseased state (1, 2). Viruses and other pathogens often induce malfunction by mimicking interaction domains of host proteins, which can rewire signaling networks and change cell responses for their own purposes (3). Frequently, viruses interact with host proteins that have many interacting partners and/or are central to many paths in the network (4), presumably because targeting these central interactions provides the most efficient means of changing host responses at a systems level. Therefore, viral infection represents a valuable model for performing systems-level analyses of signaling network function; and quantitative, systems-level approaches are needed to fully understand the mechanisms of viral pathogenicity.

The advent of high throughput and multiplex techniques, such as DNA microarrays (5), mass spectrometry (6), and highly multiparametric flow cytometry (7, 8), has enabled simultaneous experimental measurement of many components in a biological system and contributed to the growing field of systems biology. Although initially applied primarily to gene regulation and genomic analysis, systems biology studies are increasingly applied to protein networks–including cell signaling networks-due to the increased availability of high-throughput techniques to probe large protein data sets (9). It is possible to divide systems biology studies into two categories: static studies, which take a "snapshot" of a biological network under a single condition, or limited set of conditions; and dynamic studies, which measure time-dependent changes in the network following treatment with environmental stimuli or other biological cues. Both approaches have the potential to significantly increase our understanding of the complex mechanisms involved in viral infection; however, dynamic systems biology studies have been less widely pursued.

In the past few years, large-scale genomic and proteomic methods have been used to uncover the complex network of interactions that characterize viral infections of a host (Fig. 1). For example, a physical, regulatory and functional network of human-influenza H1N1 interactions constructed using a yeast two-hybrid screen and a genome-wide analysis of gene expression, led to the identification of 1735 candidate genes that might be involved in host response to influenza infection (10). Similarly, three RNAi screens of the determinants of HIV infection implicated hundreds of host proteins that were not previously identified (11-13). And very recently, microarray analysis was used to compare altered gene expression regulation in three different host cell systems following avian influenza (H5N1) infection (14). Network analyses of these data sets provide a comprehensive and organizational picture of protein-protein or genetic interactions (usually undirected) that define the cellular state following viral infection. Such maps of the virus-host interactome improve our understanding of the global nature of viral infection on the host cell's regulatory network; however they do not explain how virus–host interactions function at a systems level to affect pathogenesis.

In contrast, dynamic systems biology approaches attempt to

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describe the mechanisms of regulation between components within the system by measuring cell responses to stimuli, usually as a function of time or dose (15). The resulting data is generally context-dependent (e.g., specific to a particular cell type or condition) and multivariate, and an array of mathematical and computational modeling approaches have been used to extract underlying regulatory mechanisms (16). Measuring time-dependent responses to stimuli for defined experimental systems has been applied over the years to study the effect of viral infection or viral proteins on single signaling proteins or pathways, generating invaluable information on the mechanism of action in the network (Fig. 1). However it is often difficult to predict the effect of these interactions in the larger network. Application of dynamic systems biology approaches to virus-host signaling interactions, in which multiple signals in the network are measured simultaneously over time, may provide a better understanding of how a virus hijacks the host protein signaling network and wires signaling in favor of virus survival and replication (Fig. 1).

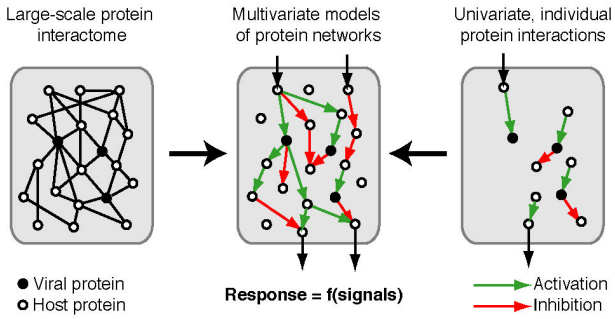

Fig. 1. Dynamic systems biology approaches to study virus-host interactions (center) complement large-scale studies of networks of virus-host protein-protein interactions (left) and molecular level studies of single pathways targeted by infection (right). In this schematic of an infected host cell's signaling network, host proteins (white circles) or viral proteins (black circles) are depicted as undirected interactions (black), activating interactions (green), or inhibitory interactions (red). Computational models (see Table 1) can be developed to understand how viral infection alters downstream signaling responses.

In this review, we will describe recent advances in quantitative, perturbation-based systems biology approaches applied to 1) defining the organization and function of virus-mediated changes to host signaling networks; 2) designing and evaluating anti-viral therapies; and 3) analysis of cell-to-cell variability and how it affects host response to viral infection. We highlight studies from both the viral and non-viral literature to demonstrate the range of computational approaches used to analyze dynamic, multivariate data to extract novel biological mechanisms. We suggest that application of these approaches to a broader range of viral infections would significantly improve our understanding of viral pathogenesis, and may uncover novel targets for antiviral therapies to treat the multitude of viral infections that continue to confound the medical community.

SYSTEMS APPROACHES TO ANALYZE VIRAL-MEDIATED CHANGES TO SIGNALING NETWORK ORGANIZATION AND FUNCTION

Viruses alter normal regulation of host signaling processes, in part by interacting with host signaling proteins to "rewire" the network (3, 17). Protein interaction networks generated from large-scale, high-throughput studies have provided topological information about the complexity of these interactions. However, such networks do not provide information about how viral infection alters host cell responses, both directly and in response to extracellular stimuli. Experimental data linking

Table 1. Glossary

Bayesian network	A form of graphical modeling that calculates the most probable set of interactions between a set of variables (e.g., proteins) based on experimental measurements of these variables
Boolean logic model	A discrete modeling technique which calculates a binary state of a protein (either "active" or "inactive") based on its dependence on the states of other proteins in the network
Cluster analysis (clustering)	A type of model that classifies objects (e.g., biological sample or conditions) based on the similarity of measured characteristics (e.g., activation state of proteins over time)
Graphical Gaussian modeling	A graphical modeling technique that calculates the dependecy of each variable on all other variables in the network
Hierarchical clustering	A subgroup of clustering analysis that arranges clusters in a hierarchical tree
Multiple linear regression	A model in which the dependent variable is a linear combination of one or more independent variables
Artificial neural network	A learning-based approach that infers a function relating an output (e.g., biological state) to input variables (e.g., protein measurements) based on a set of input-output observations
Ordinary differential equation model	A system of equations that defines the time-dependent change of each variable in the network in terms of the other variables and a set of parameters that characterize the system
Partial least squares regression	A modeling technique for relating independent and dependent variables via regression when the number of measured variables is greater than the number of experimental observations
Stochastic fluctuation	Random fluctuation due to small numbers of biological molecules (e.g., transcripts or proteins) that can lead to non-genetic heterogeneity
Superposition of effect	A model that quantifies interactions between single agents (both synergy and antagonism)

extracellular cues with downstream signals and responses (so-called "cue-signal-response" approaches) are extremely valuable for understanding which protein interactions are functionally linked to host cell responses. For example, 82 protein interactions are implicated in the immediate-early response of human cells to a range of cytokines based on literature information of the network (18). However, experimental data from time courses of signals and responses gathered in liver cells following stimulation with the same cytokine cues reveals a different picture. To assess the functional interactions evident from the cue-signal-response data, the data were fit to a Boolean logic model, which essentially adds direction of influence to protein interaction diagrams (Table 1). The resulting model had greater predictive power than the literature-derived network, while reducing the total number of connections, suggesting that many interactions found in the literature were not functional in the specific experimental system considered (i.e., liver hepatocarcinoma cells) (18). Similarly, experimental data measuring time-dependent changes in the viral systems of interest will be necessary to extract the most useful information from the viral–host protein interactome data rapidly becoming available. Below we describe a range of systems approaches that are well suited to address this challenge.

Several recent studies have directly measured time-dependent changes across multiple pathways in response to virus infection. For example, in an effort to understand why SIV infection is pathogenic in Asian macaques (a non-natural host) and not pathogenic in African green monkeys (a natural host), Lederer *et al*. measured viral-induced changes in gene expression over time and in multiple tissues derived from each host (19). The dynamic measurements showed that both infections induce a strong type I interferon response, but the interferon response peaks and falls in the non-pathogenic infection, while a sustained response is associated with pathogenesis. Another non-viral study measured a time course of global gene expression using microarrays data, and reported that infection of wild type and mutant *Salmonella* induced significantly different patterns of host signaling within phosphatidylinositol, CCR3, Wnt, TGF-β and actin regulation pathways (20). In contrast to gene expression, few studies have measured multivariate changes in signaling protein activity following viral infection. A recent pioneering investigation of host cell signaling responses to coxsackievirus B3 (CVB3) infection sampled phospho-protein dynamics in the presence of single or pairwise combinations of small molecule inhibitors (21). The signaling network reconstructed using graphical Gaussian modeling (Table 1) uncovered an extracellular autocrine circuit involving TNF and IL-1 necessary for CVB3 cardiotoxicity, and suggested a potential strategy for therapy (see below).

Another effective way to identify viral–host protein interactions that alter underlying signaling mechanisms is to measure virus-induced changes in signaling and phenotypic responses to extracellular stimuli. For example, in cells infected with an attenuated adenoviral vector (Adv), Adv infection saturated Akt pro-survival signaling and blocked insulin-mediated anti-apoptotic signaling in cells treated with TNF, causing apoptosis in host cells (2). In another study comparing HIV-1 infected and uninfected monocytes, HIV-1 infection significantly changed monocyte activation in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) via down-regulation of Jak/STAT signaling but enhancement of MAPK signaling (22), resulting in defective antigen presentation. These studies focused on a single pathway, however the extent of viral-mediated changes could be more fully understood by measuring changes across multiple pathways in the network and using computational methods to analyze the experimental data. For example, in an effort to understand system-level changes in a cell signaling network induced by cancer, secretion of 50 cytokines and measurements of 17 intracellular signals were compared between primary hepatocytes and transformed liver cell lines following stimulation with 7 inducers of inflammation, innate immunity and proliferation in the presence or absence of 7 small molecule inhibitors of specific pathways (23). The authors used multiple linear regression (Table 1) to correlate the strength of interaction between cytokine cues, signaling proteins and cytokine secretion responses, and overlaid these onto literature-constructed signaling network diagrams. This study revealed significant differences in the engagement of toll-like receptors and NF-κB dependent cytokine and chemokine release in the normal and transformed cells (23), demonstrating the underlying changes in signaling that produce the cancer phenotype. Such an approach could be analogously applied to compare virally-infected and uninfected cells.

Another approach is to compare systems-level signaling responses between viruses and environmental stimuli that act on the same network. For example, a time course analysis of genome-wide expression patterns induced in Jurkat cells in response to expression of HIV Nef or TCR stimulation with anti-CD3 demonstrated that Nef closely mimics T cell activation, and that this may prime the T cell for HIV infection (24). In another study applied to a non-viral pathogen, Franke *et al*. compared how *Helicobacter pylori* (*H. pylori*) and hepatocyte growth factor (HGF) induce activation of c-Met receptor signaling (25). The study, which applied a variation of Boolean modeling (Table 1), revealed protein targets downstream of c-Met activated only during *H. pylori* infection, but not following HGF stimulation in uninfected cells. Another recent non-viral pathogen study similarly compared growth factor- and *Salmonella enterica*-induced signaling events in host cells using a global, temporal phospho-proteomic mass spectrometry data set and clustering analysis (Table 1) (26). The authors demonstrated that changes in phosphorylation patterns in the infected cells were a result of altered Akt, protein kinase C and Pim activity, and many altered phosphorylation events depended on a single bacterial effector protein (26). Both studies thus suggest possible intervention targets for diseases induced by the respective bacterial infections, and have clear parallels

to viral infection.

Following an experimental systems analysis of how network function is altered after viral infection, the information encoded in the resulting multivariate signaling measurements can be used to build models that predict biological responses in the presence and absence of infection. The first study to demonstrate this approach in a non-viral system analyzed the molecular basis of apoptosis induction in response to combinations of both pro- and anti-apoptotic stimuli (TNF, EGF and insulin). Nineteen intracellular measurements and four distinct apoptotic outputs were quantified (27), and a partial least squares regression model (PLSR; Table 1) constructed from these data identified the underlying combination of time-dependent signals associated with apoptosis. Furthermore, the model predicted the effect of small molecule inhibition of autocrine feedback loops induced by TNF, results that were subsequently confirmed experimentally. This same type of model, based on information included in multivariate measurements of signaling responses, was able to correctly predict how infection with an adenoviral vector altered apoptotic responses to TNFα in different cell types (28). Measurements of signaling activation and apoptosis in colon carcinoma cells in response to combinations of TNF and adenoviral vector infection or IFN-γ were used to build a PLSR model that could accurately predict the increases in TNF-mediated apoptosis observed in infected cells. Surprisingly, the same model could also correctly predict how infection changed apoptotic responses in other epithelial cell types (28). The finding that a model based on multivariate signaling measurements in one cell type could predict a wide range of virus-altered responses across divergent epithelial cell lines suggests a highly promising application to a broader range of viral infection studies. Dynamic systems approaches that accurately predict cell-specific responses following viral infection could be used to test the outcomes of viral infection and anti-viral drug administration *in silico*, before pursuing different approaches experimentally.

SYSTEMS APPROACHES TO DESIGN AND EVALUATE ANTI-VIRAL THERAPIES

An improved understanding of how signal transduction pathways are altered by viral infection can be extended to reveal how manipulation of these pathways will change cellular responses to ligands or drugs, and therefore inform novel strategies for therapy. In the following studies, dynamic systems biology approaches were used to reveal network regulatory mechanisms which have significant clinical relevance.

Evaluation of drug efficacy and safety is a major endeavor in the pharmaceutical industry. Enormous effort has been directed into understanding the mechanisms of anti-viral drugs, such as anti-HCV drugs, interferon and ribavirin (29-31). However, these studies focused on how drugs affected isolated molecules or signaling pathways, and little was known about the effect on signaling pathways in the context of the larger network. In contrast, studies based on dynamic, multivariate measurements of multiple pathways can provide a comprehensive evaluation of drug action. Mitsos *et al*. monitored drug-induced signaling alterations at a systems level by measuring activation of thirteen key phospho-proteins in the presence or absence of EGFR inhibitors in a hepatocarcinoma cell line (32). By integrating their data using a Boolean-based model, they confirmed the main targets of four drugs as well as uncovered several unknown off-target effects, demonstrating an efficient way to determine drug selectivity and potency. Similarly, a Boolean model of growth factor receptor activities in response to different ligands or drugs discovered a poorly documented off target effect of TPCA-1, an IκB inhibitor (33). Similar approaches may demonstrate promising applications to investigate anti-viral drug actions.

Current anti-viral drugs generally target viral factors, such as the neuraminidase or the M2 ion channel of influenza (34) and protease, integrase or reverse transcriptase in HIV-1 (35). However, in part due to the emergence of drug-resistant strains, increasingly pre-clinical approaches focus on host cellular factors or pathways that affect virus replication (36). Given the complexity of virus–host interactions as described above, systems-level studies are well suited for evaluating such strategies. For example, recent research on novel anti-viral therapies to treat Hepatitis C virus (HCV) have focused on host sterol and protein prenylation pathways (37). To date, however, HCV therapies targeting the sterol pathway have not yielded satisfactory results. To address this problem, Owens *et al*. adopted a systems biology approach to study HCV replication in host cells treated with single or pairwise combinations of 16 chemical inhibitors that targeted the sterol and protein prenylation pathways (37). A superposition of effect model (Table 1) revealed that the causes underlying failure of the therapy were from complicated pathway regulation: sterol pathway inhibition often results in host toxicity and epistatic side effects. More interestingly, the model demonstrated that high synergistic inhibition of HCV replication can be achieved by combinatorial targeting of two downstream enzymes in the sterol pathway, revealing a potential strategy for HCV therapy.

As demonstrated by the HCV study, combinatorial drug therapy is an approach increasingly used to overcome viral drug resistance by targeting multiple factors or pathways necessary for infection (38). For the treatment of many viruses, including HIV, HCV and Influenza, combinatorial drug administration has shown increased and broadened efficacy (38-40). However, not all drugs can be combined in an effective way without increased toxicity, and therefore investigations into the combined effects of two or more anti-viral drugs are especially significant. Dynamic systems approaches combined with signaling perturbation and computational modeling offer a unique and efficient way to estimate combined drug actions. For example, a strategy called pairwise agonist scanning trained a neural network model (Table 1) based on experimental data sets of human platelet responses to all single

and pairwise combinations of input agonists (41). The model was able to accurately predict responses from combinations of three to six inputs, indicating the potential of such models to provide useful *in silico* data about combinations of physiological inputs not yet tested experimentally.

Systems biology studies can also lead to unintuitive predictions of network regulatory mechanisms during virus infection and therefore guide therapeutic strategy. Oncolytic adenovirus has been tested in clinical trials for cancer therapy, but efficacy has so far been inadequate. Inhibition of MEK has the potential to enhance adenovirus infection but also decreases adenovirus replication, making it unclear how to optimize this strategy (42). To resolve this dilemma, Bagheri *et al*. constructed an ordinary differential equation model (Table 1) based on virus receptor expression, cell viability and proliferation, and virus infection and replication in order to test various treatment strategies (42). Modeling dynamic cancer cell activities in response to MEK inhibition and adenovirus infection predicted that optimal oncolytic treatments could be achieved by simultaneous treatment with oncolytic adenovirus infection and MEK inhibitors.

Another common problem leading to unexpected consequences of therapy, both in viral infection and other diseases such as cancer, is the existence of autocrine loops, in which secreted cytokines induce unexpected responses downstream of an initial stimulus. In a study on TNF-induced human epithelial cell signaling (43), a classifier based regression model built on ~8,000 intracellular measurements revealed that TNF, a pro-death stimulus, activated a sequential release of three cytokines, both pro- and anti-apoptotic in function. The signaling induced by the autocrine cascade specified the extent of apoptosis induced by TNF. This study provided insight into why certain anti-tumor drugs targeting only one component of this response are sometimes ineffective (43). Autocrine circuits similarly confound effective treatment strategies for coxsackievirus B3 (CVB3) infection (see above). The same systems biology study that uncovered an extracellular autocrine circuit involving TNF and IL-1 necessary for CVB3 cardiotoxicity, demonstrated that blocking this positive feedback circuit significantly inhibited CVB3 replication and improved host cell viability (21). These discoveries have important parallels in host-viral systems as several viruses have been reported to interact with TNF/TNFR pathways to favor virus replication (Epstein-Barr virus) or trigger the killing of bystander cells (Hepatitis B virus and HCV) (44). Thus, future investigations of the role of extracellular autocrine circuits in host-viral systems may provide invaluable insights on viral pathogenesis and the development of efficient anti-viral therapy.

SYSTEMS APPROACHES TO ASSESS THE EFFECT OF CELL-TO-CELL VARIABILITY IN VIRAL INFECTION

One question facing much of biology is why cells exposed to the same stimuli or pathogen demonstrate different phenotypic outcomes (45). Significant effort has been and continues to be made in understanding how cells integrate and respond to environmental perturbations at a population level. However, population-averaged measurements often fail to recognize important information that originated from population heterogeneity. In the past, cell-to-cell heterogeneity was often considered an obstacle to interpreting population responses induced by extracellular stimuli. However, quantitative systems analyses of signaling networks at a single cell level are using the information encoded in cell population heterogeneity to yield valuable insights into understanding signaling network function, including in response to viral infection.

Cell-to-cell heterogeneity is seen at all levels of the viral lifecycle, including 1) heterogeneity in the incidence of infection itself (46); 2) variation in host cell response following infection (47); and 3) heterogeneity in viral fate, such as replication versus latency (48, 49). To understand cell-to-cell variability in viral infection, Snijder *et al*. collected large numbers of single-cell measurements of each cells' local environment (e.g., cell population size, local cell density, single cell position, cell size, mitotic state and apoptotic state) from three types of cells infected by one of three viruses (46). Using graphical Gaussian modeling and linear regression (Table 1), the researchers built a model that was able to predict heterogeneous infection patterns based on each cells' population context. Moreover, Bayesian network learning (Table 1) revealed a novel infection mechanism for SV40 virus, which was further validated by experiments. This study not only revealed how heterogeneity of cellular activities during virus infection accounts for variability in infection, but also demonstrated how systems biology approaches at the single cell level can improve understanding of viral infection mechanisms.

Viral infection can also induce cell-to-cell viability in activation of signaling pathways. A recent study in mouse cells infected with Sendal virus demonstrated that cells infected with similar levels of virus showed stochastic expression of interferon-β (IFNβ) and other virus-inducible genes (50). The reason for the stochastic IFNβ response appeared to be due to cell-to-cell variability in host protein factors mediating all levels of the infection process, including sensors of infection, signaling proteins, and transcription factors (50). Interestingly, naturally-occurring variations in protein levels were also shown to account for cell-to-cell heterogeneity in apoptotic responses to treatment with TRAIL (51). This suggests that resistance to treatment exhibited by many cancer cell populations may not be genetically based, a result that may also apply to anti-viral treatment. Such natural stochastic fluctuations (Table 1) may also account for observed differences in cell fate, such as the replication-versus-latency decision of HIV-1 (48, 49). Stochastic fluctuations in the viral protein Tat were shown to result in different patterns of gene expression in populations of Jurkat cells clonally infected with a minimal HIV vector (52, 53). Whether or not variability in host proteins also contributes to this viral fate decision remains to be worked out.

Measurements of signaling proteins at the single cell level increasingly reveal network functions and mechanisms that are masked in the population-level measurements. For example, uniform stimulation of a PC12 cell population by nerve growth factor (NGF) causes some cells to differentiate and some cells to proliferate. A two-dimensional phospho-ERK-phospho-Akt single cell response map constructed by single cell imaging analysis of cells treated with NGF revealed a distinct boundary separating differentiating and proliferating cells (54). The same pERK–pAkt boundary separated proliferation-versus-differentiation fate decision in response to other growth factors, which suggested a mechanistic strategy by which cells regulate differentiation within the population (54). Interestingly, another recent study exploited the cell-to-cell variability of viral infection to quantify signaling responses to heterogeneous levels of MYC protein (55). By using single cell imaging to quantify signal activation in response to a wide range of overexpressed MYC delivered by an adenoviral vector, the authors revealed a biphasic pattern in the activation of MYC's downstream effector, E2f1, accounting for conflicting reports of the effect of MYC overexpression.

Increasingly, single cell studies at a systems level are revealing the complexity of signaling responses in cells of the immune system. Given the close relationship between viral infection and the resulting host immune response, these studies may provide important insights to improving anti-viral therapies and vaccine development. For example, a serial, time-dependent, single cell analysis of IFN-gamma, IL-2 and TNF-alpha secretion by primary human T cells in response to activating stimuli showed that cytokine release is asynchronous and that the majority of cells secrete only one cytokine at a time (56). Furthermore, the study showed that T cells release cytokines following a programmatic pattern which is associated with the differentiated state of the cell. A similar study applied information theory to investigate fluctuations in protein secretion from single human macrophage cells in response to LPS. By characterizing the fluctuations of different secreted proteins in a single cell and in small cell colonies, the authors were able to construct a protein-protein interaction network and quantitatively predict the role of perturbations (57). Notably, both of these studies were conducted in microfluidic devices, which increasingly facilitate quantification of signaling and cytokine responses in single cells due to the sensitivity provided by miniaturization of assays (58).

Single cells from the same viral-infected population exhibit differences in the number of produced virus particles spanning over 300-fold (59), and this diversity will surely impact infection dynamics and treatment strategies. Therefore, studies of virus–host interactions will be especially enhanced by the growing availability of multiplexed experimental measurements of proteins in single cells, and computational approaches to derive biological understanding from this multitude of data.

CONCLUSIONS

Viral infection is a systems-level perturbation, and therefore systems biology approaches are naturally suited to studying the complexity of viral pathogenesis. We have highlighted a number of innovative examples from both the viral and non-viral literature, which demonstrate the potential for dynamic systems biology signaling studies–characterized by time-variant, context-dependent responses to viral infection–to complement both molecular, single pathway efforts and high-throughput, large-scale analyses of viral–host interactomes (Fig. 1). We expect that systems biology approaches designed to define signaling network organization, discover novel regulatory mechanisms, and predict phenotypic responses to viral infection will uncover many novel mechanisms underlying viral pathogenesis and point to promising strategies for anti-viral therapy.

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