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<td>Selvarajoo, Kumar</td>
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Abstract: The recent systems biology efforts are revealing complexities beyond traditional static causal relationship understanding. Our research has focused on dynamic cellular behaviors to uncover novel network and global response properties. We investigated instructive cell signaling and high-throughput transcriptome-wide behaviors of immune, cancer, and embryonic development cells. Our data reveal that cultured populations display deterministic signaling that can be modeled using linear response rules. Adopting the rules, we have computationally predicted and verified experimentally novel signaling features and key targets for controlling proinflammatory and cancer responses. In addition, our transcriptome-wide statistical analyses of macrophage immune response and single cell embryonic developmental process, from oocyte to blastocysts cell stage differentiation, revealed interesting global patterns where even lowly expressed genes play a role.

Keywords: cell signaling, cancer, immune, development, systems biology
1 Introduction

Living organisms are highly complex dynamical systems that are able to survive, proliferate, and evolve under variable conditions for thousands, if not, billions of years. Over their evolutionary period, each living species has been able to detect, process, respond, and adapt to different environmental information. The immune system is one such mechanism by which living systems are able to neutralize potential threats by detecting external agents and invoking appropriate intracellular response for protection. Living systems are not only able to defend themselves individually, in many instances they are able to pool together, or self-organize, to protect the entire population. Thus, they are not only complex within their own physiology, but are also sophisticated in their dynamical cooperative behavior in adaptation to their surroundings.

For the past decade, our research has been focused on understanding the complex and dynamic perturbation-response of cellular systems. In particular, we have centred on cell signaling or the tracking of extracellular stimuli affecting numerous intracellular molecules to trigger the transcription of genes for myriad cellular processes such as differentiation, proinflammatory response and apoptosis. These processes are vital to investigate as they not only provide understanding of the normal functioning of living cells, but may also provide clues on disease occurrence or targets for disease control.

In this paper, I will discuss our research, adopting systems biology approaches, to understand and tackle proinflammatory responses of the immune and cancer cells, and shed some insights into the complexities of embryonic stem cell differentiation. Our strategy involves utilizing mathematical, computational, and statistical approaches to analyse dynamical cellular datasets, from low-throughput western-blot, enzyme-linked immunosorbent assay (ELISA)
or electrophoretic mobility shift assay (EMSA) readouts, to high-throughput gene expression profiles derived from DNA microarrays and next generation (RNA) sequencing techniques. Using cross-disciplinary approaches, our aims were to better understand complex network behaviors and their governing principles, and using the derived knowledge to systemically determine novel targets for more optimal regulated response. In the following sections, I will provide details of our goals and achievements.

2 Cell Signaling in Immune Response and Cancer

Immune diseases and cancer deaths still remain a major global challenge affecting people from all walks of life. Despite the vast amount of time, efforts and funds put to challenge the diseases worldwide, medical treatments remain suboptimal and are largely unchanged over the past 40 years. To tackle some of the difficulties facing current research, we undertook an integrative, multidisciplinary approach for investigating the mammalian innate immune response and its link to cancer.

3 Uncovering Novel Features in Toll-like Receptor Signaling

We initially, about a decade ago, began our research on the innate immune response invoked by the Toll-like receptors (TLRs). TLRs are transmembrane proteins that function to recognize conserved pathogen-associated molecular patterns (PAMPs) related to microorganisms, such as lipopolysaccharide (LPS) from gram-negative bacteria and double-stranded RNA (dsRNA) from viruses. There are 13 known members of the TLRs in mammals. TLRs 1, 2, 4, 5, 6 are located at cell surface, while TLRs 3, 7, 8, 9, 11, 13 are bound to the intracellular endosomes. Each TLR recognizes specific PAMPs and trigger microbial clearance and induce the production
of immunoregulatory chemokines, cytokines, and cell surface and costimulatory molecules. The TLRs also increase effector functions such as phagocytosis, and present antigen to adaptive immune cells \[1\]. Thus, the activation of TLRs is a first line of mammalian’s immune defense system.

The most well characterized PAMP is the TLR4. Upon LPS binding, TLR4 triggers two major intracellular pathways, the MyD88- and TRAM- dependent pathways. The MyD88-dependent pathway mainly induces proinflammatory cytokines such as TNF, IL-6, and SOCS3 through activation of MAP kinases p38, ERK, JNK and NF-κB \[2\]. The TRAM-dependent pathway, on the other hand, predominantly induces type I interferons (IFNs) and chemokines such as IP-10 (encoded by \(Cxc\text{lix}10\)) and interferon (IFN)-induced proteins through activation of IRF -3 or -7 and NF-κB. Thus, both pathways complement each other in the production of pro-inflammatory mediators. Although there are detailed experimental works on TLR4 signaling, the dynamical response of the MyD88- and TRAM-dependent pathways remained poorly understood.

We investigated the TLR4 response \[3\] in wildtype and several mutant conditions using a computational model based on the perturbation-response approach (Box 1), followed by experimental verification. Our initial task was to investigate molecular mechanisms for the impaired and delayed kinetics of NF-κB activation in MyD88 knock-out (KO) murine macrophages \[4\]. According to our modeling approach, the impairment was due to lower signaling flux (affinity) towards the TRAM-dependent pathway, compared with MyD88-dependent pathway, in LPS stimulation and the delay in NF-κB was due to several unknown signaling intermediates or process acting upstream of TBK in the TRAM-dependent pathways (Fig. 1A). In addition to these, the model also predicted delayed induction of TNF
and enhanced expression of Cxcl10 in MyD88 KOs (Fig. 1B).

To validate the model predictions, we subsequently performed experiments on murine macrophages \[^5\]. Firstly, we proved the delayed induction of Tnf and enhanced expression of Cxcl10 in MyD88 KO murine macrophages (Fig. 1C). Next, we showed the mechanism for the enhanced activation of Cxcl10 in MyD88 KOs is due to signaling flux redistribution or SFR (Fig. 1D). The experiment also proved the point of lower signaling flux (affinity) towards the TRAM-dependent pathways in the absence of MyD88, when TRAM preferentially bound to intracellular TLR4 domain (Fig. 1E). Notably, the speculation that the TRAM-dependent pathway consisted of additional signaling intermediates or processes was confirmed by later studies, demonstrated by the sequential activation of CD14, ITAM-mediated process of tyrosine kinase Syk and its downstream effector PLC\(\gamma\)2 for the endocytosis of TLR4 prior to TRIF/TRAM activation \[^6, 7\], and the phosphorylation of TRAM by PKC\(\varepsilon\) for IRF-3 activation \[^8\].

4 Systems Biology to Regulate TNF Signaling

Following the successful prediction and validation of our TLR4 model, we next embarked on studying the tumor necrosis factor (TNF) signaling. As noted above, TNF is one of the key cytokine induced by the proinflammatory response of TLR4. TNF, which is also produced by various other signaling cascades, plays a major role in regulating myriad cellular processes \[^9\]. Chronically elevated levels of TNF have led to several major illnesses including rheumatoid arthritis and certain types of cancers \[^10, 11\].

We embarked on the development of a new TNF signaling model, and using the model intended to find a key optimal target that will selectively and effectively suppress, but not abolish, TNF-induced proinflammatory response \[^12\]. This is because, total abolishment of
Conceptualising Cell Signaling and Transcriptome-wide Response for Targeted Experimentations

Fig. 1(A)

Fig. 1(B)

Fig. 1(C)
TNF response will immuno-compromise subjects to pathogenic threats.

First, we curated the literature and theoretically derived the TNF signaling topology (Fig. 2A, black arrows). A dynamic mass-action model (Box 1) was developed based on this topology with parameter values chosen to fit the temporal profiles of p38 and IκBα in wildtype and several mutant conditions (Fig. 2B). Subsequently, we extended the model to simulate 3 major groups of upregulated proinflammatory genes in TNF stimulation (Fig. 2C).

It is important to emphasize that our computational models fitted to the wildtype experimental data often failed to recapture
profiles in mutant conditions. The main reason for this, to our knowledge, is the general lack of detailed network information of our signaling topologies. To overcome this limitation, we have developed 9 response rules that will help guide us to correct any grossly missing information. Note that our works, and that of others in related studies, have demonstrated that biological networks are often sensitive to network topology rather than parameter values. Hence, the use of response rules (Box 2) to modify and re-investigate signaling topologies is an appropriate next step.

For the TNF signaling, although our model was able to fit both
Figure 2  (A) Schematic of TNF signaling with modified novel feedback mechanisms in grey arrows. (B) Temporal experimental profiles (left) and dynamic computational simulations (right) of $I\kappa B\alpha$ (top) and $p38$ (bottom) activity in wildtype (black) and various KO conditions (colors). (C) Average expression profiles of genes in groups I (red), II (green), and III (blue) in 3T3 fibroblasts stimulated with TNF. Simulation profiles of the 3 groups of genes using initial TNF model (D) and modified model (E) with transcriptional delay and novel feedback mechanisms (solid lines) or with transcriptional delay and without feedback mechanisms (dotted lines). (F) Simulation profiles of the 3 groups of genes in wildtype and 3 key KO conditions ($I\kappa B\alpha$ KO, RIP1 KO, and TAK1 complex KO) using the modified model with feedback (note that for all other KO simulations, see [12] for details). (G) Temporal gene expressions of groups I ($Tnfaip3$, $I\kappa B\alpha$, $Jun$, $Nfkbia$), II ($Ccl7$, $Vcam1$, $Cxcl10$), and III ($Mmp3$, $Mmp13$, $Enpp2$) genes in TNF-stimulated BALB/3T3 (top) and MEF (bottom) cells, treated without (dark color) and with (light color) Nec-1. (A) reproduced from [40] and (B-G) adapted from [12].
p38 and IκBα in wildtype and several mutant conditions, when the model was extended to simulate gene expressions, the wildtype model was insufficient to fit group 3 gene profile: the simulation clearly underestimated the transcription levels after 2 h (Fig. 2D). According to our response rules (Rules 2 & 4, Box 2), a post-transcriptional feedback mechanism, specifically to IkappaB-alpha, is required for the continuous activation of group 3 genes. Adding this hypothesis to the model resulted in significant improvement to the dynamical simulations (Fig. 2E).

Next, we tested, in silico, the effect of down-regulating all signaling proteins in the TNF topology for controlling the expression of the 3 major groups of proinflammatory genes which are usually upregulated in proinflammatory diseases. Among the data, we found RIP1 knock down (KD) simulations produced moderate regulations of all 3 groups of genes (Fig. 2F). This data indicated that RIP1 may be an attractive target for controlling TNF-induced proinflammatory response. Hence, to validate this important result, which may provide significant benefit for proinflammatory therapeutics, we performed actual experiments on 2 cell types (MEF and 3T3).

Necrostatin-1, or Nec-1, is a well-known specific inhibitor of RIP1[19]. We compared 10 proinflammatory gene expressions in MEF and 3T3 cells stimulated with TNF, with and without Nec-1 pretreatment (Fig. 2G). The experimental results confirmed our model simulations, and also demonstrated that Nec-1 could potentially be used to treat proinflammatory diseases such as rheumatoid arthritis or osteoarthritis.

5 Enhancing Apoptosis by Tinkering TRAIL Signaling

Cancer cells are highly variable and largely resistant to therapeutic intervention. The TNF-related apoptosis-inducing ligand
(TRAIL) induces apoptosis in malignant cells, while leaving other cells mostly unharmed. However, several carcinomas remain resistant to TRAIL \[1\]. To investigate the resistance mechanisms in TRAIL-stimulated human fibrosarcoma (HT1080) cells, we developed a computational model to analyze the temporal activation profiles of cell survival (IκB, JNK, p38) and apoptotic (caspase-8 and -3) molecules in wildtype and several (FADD, RIP1, TRAF2 and caspase-8) KD conditions \[20\].

Our initial model was based on the well-known TRAIL signaling (Fig. 3A-2, black arrows). Similarly to our TLR4 and TNF works, the initial computational TRAIL model could only fit the wildtype dynamical activation profiles of the 5 signaling molecules (IκB, JNK, p38, caspase-8, and caspase-3), and failed to match mutant conditions. Next, by carefully applying the response rules (Box 2), the TRAIL signaling topology was modified in silico step by step until all tested molecules matched the 4 available mutant conditions (RIP1 KD, FADD KD, caspase-8 KD, and TRAF2 KD) (Fig. 3A-1). As a result, two novel molecules have been predicted by the revised model, i) molecule \(Y\) acting independent of FADD and able to activate JNK and p38, and ii) molecule \(Z\) specifically activating JNK via p62 (Fig. 3A).

Using the revised TRAIL model, we next investigated which of the two novel molecules is key to suppress cell survival activation or increase cell death pathway activity. Notably, the simulations suggested that knocking down molecule \(Z\) will significantly increase apoptosis and may result in 95% cell death (Fig. 3B). In other words, knocking down or inhibiting molecule \(Z\) in TRAIL-stimulated human fibrosarcoma will likely sensitize the large majority of resistant cells to death. To identify molecule \(Z\), we performed a search on the protein-protein interaction database for p62 interacting partners, and obtained protein kinase C (PKC) family members as likely candidates. However, which PKC member might be molecule \(Z\) remained to be identified.
Consequently, we utilized PKC inhibitor, bisindolylmaleimide I (BIM-I), to experimentally verify whether TRAIL-stimulated HT1080 cells will indeed significantly induce apoptosis in the presence of PKC inhibition \cite{21}. In addition to HT1080, we also tested another TRAIL-resistant cancer cell, the human colon adenocarcinoma (HT29) cells. Notably, as predicted by the computational model, PKC inhibition during TRAIL stimulation produced over 95% cell death for both HT1080 and HT29 cells, with relatively insignificant effect on normal control TIG-1 and MRC5 cells (see Fig. 1 of ref. 21). These results confirmed our model simulations that a PKC family member is
Figure 3  (A) Schematic of TRAIL signaling with modified novel pathways in grey (A2), and simulation profiles (curves) compared with experiments (shapes) of p38, IκB, JNK, caspase-8 and -3 in wildtype, RIP1 KD, FADD KD, caspase-8 KD, and TRAF2 KD (left) for TRAIL stimulated HT1080 cancer cells(A-1). (B) Survival ratios, experimental (dark grey) and evaluated based on simulations (light grey), for all conditions including novel molecules Y and Z KD for simulations. (C) Experimental survival ratios for increasing levels of TRAIL stimulation with and without increasing doses of PKC inhibitor (BIM-I) in HT1080 cells. (A,B) taken from [20] and (C) adapted from [21].
molecule \( Z \), and its inhibition significantly sensitizes TRAIL-induced cell death (Fig. 3C).

Next, to identify which PKC member, among 10 known, is molecule \( Z \), we tested all of them experimentally. Notably, we found that PKC\(\delta\) siRNA produced the same amount of cell death at 3h compared with BIM-I treatment (see Fig. 4C of ref. 21). Thus, we concluded our study by revealing that specific PKC\(\delta\) inhibition in TRAIL-based therapy has great prospects for malignant cancers that are resistant to TRAIL \(^{[21]}\).

Overall, from our systems biology research of using dynamic computational modeling to identify novel cell signaling mechanisms or targets, and subsequently performing experiments to verify them in TLR4, TNF, and TRAIL signaling has been highly successful. We believe our modeling successes are not through “chance”, but demonstrate the presence of simple rules guiding complex behaviors through physical laws on a macroscopic top-down scale \(^{[1,14,22]}\). Our data provide further evidence for the utility of systemic approaches to tackle and treat complex diseases more effectively. In the following section, our other works on high-throughput omics data will be discussed.

6 Transcriptome-wide Analysis

The previous sections on cell signaling were based on a limited set of signaling proteins and genes. Although the findings shown are promising, a living cell actually contains several thousands of genes, proteins, and metabolites. To grasp the complex large scale system-level properties, the development and analysis of high-throughput experimental technologies for genomics, proteomics, and metabolomics are required. Over the last two decades, these methods have been intensively investigated, and today they generate large quantity of biological data at different scales crucial for unraveling the detailed
molecular composition and complexity of living organisms.

For instance, what types or kinds of genes are induced by the immune cells in response to invading pathogens? In a study to investigate the dynamic response of innate immune cells (macrophages) exposed to pathogenic agents (LPS), cDNA microarrays revealed that almost 3000 genes were expressed over a period of 24 hours\textsuperscript{[23]}. Apart from immune-related genes, genes belonging to diverse cellular processes were also expressed. Thus, the study was one of the first to show, from a global analysis, that a single receptor stimulation can result in the response of thousands of genes not directly related to the original function. Such valuable information suggests that high-throughput analysis of biological components are crucial for providing another dimension (of scale) to the understanding of complex cell behaviors.

7 Global Immune Response

From the second half of last decade, we have developed simple statistical techniques to investigate high-throughput gene expression dataset\textsuperscript{[24-30]}. Instead of using arbitrary threshold cut-off to eliminate genes of very low expressions (or high signal-to-noise ratio), which often results in removing 90% of genome data, we used power-law, correlation and noise approaches to analyze the entire global response patterns. Such statistical techniques have been widely used to investigate deterministic patterns of highly noisy data of other complex systems such as the weather\textsuperscript{[31]}, stock markets\textsuperscript{[32]}, and cosmology\textsuperscript{[33]}

We examined the time-series genome-wide (22,690 Affymetrix-based microarray probes) response of LPS stimulation (TLR4 signaling) in wildtype and 3 mutant conditions (MyD88 KO, TRIF KO, and MyD88/TRIF Double KO) of murine macrophages\textsuperscript{[24]}. The aim of
the study was to compare the global gene expression patterns invoked by wildtype and the mutant innate immune cells (macrophages) to environmental threat (LPS). We do recognize the fact that microarray or even the recently developed RNA-Seq datasets are prone to a large degree of technical error or biases, especially for the lowly expressed genes. Nevertheless, our goal was not to specifically identify individual novel genes expressed in all four conditions. Instead, we examined the global collective behaviors of the LPS-induced innate immune response.

Pearson correlation analysis\(^{[28]}\), which provides a measure of deviation from unity as a source of difference between the samples was adopted. In our case, the Pearson correlation coefficient shows the compressed (averaged) information of the genome-wide response. We developed a scheme to compare the correlation coefficients between (i) the same genotype at different times (e.g., wildtype 0 h vs. wildtype 1 h, called *auto-correlation*) and, (ii) the same time point with different genotypes (e.g., wildtype 1 h vs. MyD88 KO 1 h, called *cross-correlation*).

From the correlation plots, DKO auto-correlations were surprisingly similar to single KOs on the temporal scale (Fig. 4A, top). This data indicated that LPS is able to invoke gradual intracellular response, as seen by the monotonic deviation of correlation coefficient from unity, independent of the key adaptor signaling molecules of MyD88 and TRIF. The cross-correlations revealed that DKO response, compared with wildtype, is the least similar (Fig. 4B, top). This result indicated that DKO genome response, compared with single KOs, is most distinct from the wildtype response. Overall, although impaired, it was surprising that DKO, previously known to abolish all significant gene expressions\(^{[34]}\), is able to invoke global gene expression response.
To confirm whether DKO induces genome response, correlation coefficients of whole genome were compared with an ensemble comprising only of well-known proinflammatory genes (Fig. 4A,B, bottom). Notably, for the selected group of 157 proinflammatory genes, the auto-correlation of DKO was almost unchanged with time, indicating no noticeable ensemble response, consistent with other studies \(^{34, 35}\). Thus, these results indicated the presence of novel pathways, independent of MyD88 and TRIF, to activate novel gene expressions in DKO. Although we had pointed out a few biological processes not specifically related to immunity using the Gene Ontology database, we were unable to experimentally verify the specific DKO or TLR4-independent response of LPS at that time.

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**Figure 4** (A) Auto- and (B) cross- correlations of whole genome (top) and 157 proinflammatory or immune genes (bottom) after LPS stimulation. x-axis represents time and y-axis represents the correlation coefficients. Figure obtained from \([24]\).
Nevertheless, recently, two research teams have identified TLR4-independent LPS activation of caspase-11, which plays a pivotal role in shaping inflammasome \cite{36, 37}. Thus, our temporal Pearson statistical analysis was able to predict novel regulatory response from genome-wide expression dataset with further experiments required for confirmation.

8 Noise in Human Developmental Cell Differentiation

The cell population responses, dealt so far, have been instrumental not only for cell signaling, but also for large-scale or global understanding of myriad deterministic biological processes such as immune response, growth, and metabolism. However, each cell within a population is not identical in its morphology or shape, and the intracellular molecular environment is highly inhomogenous. Furthermore, even genetically identical cells produce diverse phenotypes, such as a single stem or progenitor cell can produce distinct lineages, which can be tilted even by small external perturbations. Population-wide average techniques (e.g. linear response models) are not suitable for the investigation of cellular variability and inhomogeneity \cite{38, 39}.

Recent single-cell experimental techniques have revealed fluctuations in gene and protein expressions over time. Such fluctuations, measured by transcription, phosphorylation, morphology, and motility, have been key for generating cellular heterogeneity. Increasingly, investigators recognize that a combination of intrinsic and extrinsic factors contribute to cellular stochasticity: i) intrinsic or ‘uncorrelated’ noise; the random nature of biochemical reactions, e.g. due to low copy numbers of intracellular molecules in a Poisson process, and ii) extrinsic or ‘correlated’ noise; non-Poisson fluctuations in other cellular components or states that indirectly
affect the expression of a specific gene or protein.

To understand global gene expression structure and noise patterns of single cells during mammalian developmental stages, we investigated transcriptome-wide RNA-Seq expressions of human and mouse developmental cells. A total of 7 in human and 10 in mouse, from oocytes to blastocysts, were analysed using correlation metrics, Shannon entropy and noise analyses.

Using gene expressions scatter plots, Pearson correlation and Shannon entropy between single cells data, we observed sharply increased variability from 2-cell to 4-cell stage onwards in both human and mouse (Fig. 5A). Next, global noise of single cells was investigated by quantifying the squared coefficient of expression variations over mean expression values which showed transition of noise patterns occurring between 2-cell and 8-cell stage (Fig. 5B).

To understand the possible mechanisms for increased noise patterns for 4-cell stage onwards, we developed a stochastic transcriptional model based on ordinary differential equations and fitted the model to experimental noise patterns (Fig. 5C). From the simulation results, we concurred that the early developmental stages were mainly dominated by low transcriptional activity dominated by Poisson noise. The increase in transcriptome-wide noise for the middle stage developmental cells was due to stochastic transcriptional amplification, which generated heterogeneity in gene expressions between individual cells. Such heterogeneity has been shown to be necessary for cell fate diversifications (see review in ref. 38). For the later stages, on top of high transcriptional process, the cells possess quantal activation of most transcription factors, or are subject to more extrinsic variability such as phenotypic diversity among individual cells. These factors increase the general expression scatter and noise levels.
Fig. 5(A)
Overall, the investigations into the transcriptome-wide expressions of the early mammalian developmental stages revealed increasing variability and noise patterns across the mammalian development process. These data suggested different stages of the cell differentiation process can be better understood by investigating the transcriptome-wide noise patterns. To summarize, our systemic approach provided novel insights into the transcriptome-wide expression and noise patterns for development cells, and the underlying nature of the transcriptional mechanisms.

In conclusion, in this paper, our previous works on specific...
instructive cell signaling in immune and cancer response, as well as, the global gene expression analyses of immune and developmental cells have been reviewed. Our multidisciplinary techniques have allowed the elucidation of novel properties of dynamic cell behavior, both from deterministic and stochastic perspectives, not possible using traditional low throughput and steady-state data of molecular species. In coming years, we expect to witness further growth in complex systems biology approaches.

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References

Box 1: Perturbation-Response Approach

The perturbation-response approach involves giving a small perturbation to the concentration of one or more reactant species in a network and analyzes the response profiles of other species within the network [1, 13-16]. To briefly examine, consider a linear-chain of reactions (X1 → X2 → X3 → ... ) at steady-state condition. If the concentration of X1 is pulse perturbed, the concentrations of X2, X3, etc., will increase, go through a maximum, and then decrease back to its steady-state value in sequential order (see Fig. 2.2 of ref. 1). The experiments, based on the law of information conservation, connect the species between input and output fluxes through a linear superposition of propagation response waves (first-order response) [1, 13-16].

Despite the simplicity of the approach, linear response is visually apparent in the glycolytic metabolite profiles, EGF (epidermal growth factor), TLR3, and TLR4 signaling dynamics (see Fig. 2.3 of ref. 1). Although the kinetics could vary slightly from sample to sample, the general average response profiles are very well reproducible. In other words, regardless of how complex a signaling topology might be, the species’ average dynamic responses followed deterministic formation and depletion waves [1,13,14,16]. Notably, it can be shown, theoretically, that no matter how complex or non-linear the signaling system is, the dynamic response can be approximated using first-order terms if the perturbation levels are small [1,13,14,16].

Unlike typical kinetic models, which often use similar equations or sometimes with non-linear expressions to model the dynamics of biological networks, our perturbation-response approach considers the network as a sequence of events rather than molecules. As signaling
networks are largely not fully understood, this difference is crucial as it prevents rigidly fixing the network topologies, and allows it to be modified according to experimental data so as to prevent overfitting problems and to identify novel features of signaling networks. In addition, as signaling process involves large number (thousands) of intracellular molecular activations, it is currently not plausible to model the dynamics of all possible reactions with the generally limited data. To overcome such difficulties, our approach permits the lumping of several molecules into a signaling specie in the model network. In this way, although the model does not become a comprehensive representation of an entire signaling process, however, it still allows the identification of overtly missing key features.

To successfully identify novel features of signaling networks, we set a target that the computational model should be able to simulate not just one experimental condition (like most models do), but in as many conditions as available (see maintext). Like any other modeling approach, there are certain limitations that require mentioning. Firstly, the perturbation-response approach discussed does not comprehensively represent the details of each signaling reaction’s kinetics. Secondly, the small perturbation assumption leading to the first-order mass-action equations represents an average cell response and this cannot be used to study single cell stochastic behavior or non-linear behaviors such as bistability. Thirdly, the model predictions will show relative, and not absolute, activation levels. However, the approach can be universally applied to model any pathways that experimentally display formation and depletion waves.

Box 2 Response Rules

**Rule 1, Controlling flux:** Controlling the upstream parameter of a hypothetical molecule X2 (See Fig. S1 of Ref. 12) mostly affects the slope of the formation part of the expression profile. Alternatively, controlling the downstream parameter mainly modifies the expression profile’s depletion part. **Rule 2, Time delay:** by comparing the time to reach peak activation, any time delay in target signaling molecule’s activation represents ‘missing’ cellular features such as directed transport machinery, protein complex formation, and novel molecular interactions. **Rule 3, Feedforward flux:** A) Rapid kinetics: when simulation of a downstream molecule is noticeably quicker than experimental dynamics, B) Similar kinetics: when removing a molecule along a pathway does not completely abolish its downstream intermediates, C) Delayed kinetics: when removing a molecule along a pathway show significant delay. In all these cases, the superposition principle suggests a novel feedforward pathway with different number of intermediates. **Rule 4, Feedback flux:** when a response profile shows multiple peaks or continuous increase of activation not following pulse perturbation response, this indicates feedback pathways such as posttranslational effect or secondary (autocrine/paracrine) signaling. **Rule 5, Signaling Flux Redistribution (SFR):** At pathway junctions, removing a molecule enhances the entire alternative pathways. **Rule 6, No SFR:** At pathway junctions, removing a molecule does not enhance the alternative pathway, suggesting novel i) intermediate(s) between the removed molecule and the pathway junction or ii) pathway link between the removed molecule and the alternative pathway. **Rule 7, Differential flux:** quantifies each pathway branch by comparing activation levels between wildtype and mutants data. **Rule 8, Reversible flux:** when a response profile show limiting decay that cannot be modeled by first-order decay, the presence of reversible step is expected to produce limiting decay. **Rule 9, Non-linearity:** When complex dynamics is observed, the linear response approach breaks down, and non-linear approaches are needed.

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