Mathematical Modeling and Evolution of Signal Transduction Pathways and Networks

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Declaration

I, Mohammad Mobashir, hereby declare that the work contained herein has been created independently and has not been submitted elsewhere for any other degree or qualification. The research work was carried out from August 2010 to May 2013 at the institute of Molecular and Clinical Immunology, Otto-von-Guericke University, Magdeburg.

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Abbreviation

STNs:	Signal Transduction Networks
SNs:	Signaling Networks
RSNs:	Random Signaling Networks
SMs:	Signaling Molecules
ODEs:	Ordinary Differential Equations
MAPK:	Mitogen Activated Protein Kinase
TCR:	T Cell Receptor
ITAM:	Immunoreceptor Tyrosine-based Activation Motif
LCK:	Lymphocyte protein tyrosine kinase
DAG:	Diacylglycerol
IP3:	Inositol triphosphate
GADS:	Grab2-related Adapter Protein Downstream of Shc
ITK:	Inducible T cell Kinase
PIP2:	Phosphatidylinositol 4,5-biphosphate
PLCγ1:	Phospholipase C gamma-1
NF-κB:	Nuclear Factor kappa-light-chain-enhancer of activated B cells
ER:	Endoplasmic Reticulum
CRAC:	Calcium Release Activated Ca^{2+} Channel
PKB:	Protein Kinase B
PDK1:	Phosphoinositide-Dependent Kinase-1
NFAT:	Nuclear Factor of Activated T cells
SLP76:	SH2 domain containing leukocyte of 76kDa

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List of Publications

1. **Mobashir M**, Schraven B, and Beyer T (2012) *Simulated evolution of signal transduction networks*. PLoS ONE.December 7(12): e50905.**PMID: 23272078**.

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ABSTRACT

Signal transduction is the process of routing information inside cells when receiving stimuli from their environment that modulate the behavior and function. In such biological processes, the receptors, after receiving the corresponding signals, activate a number of biomolecules which eventually transduce the signal to the nucleus. The main objective of my work is to develop a theoretical approach which will help to better understand the behavior of signal transduction networks due to changes in kinetic parameters, network topology, and the concentration of the signaling molecules for different values of input signal. Additionally, I have also investigated the role of different possible cross-talks on the nature of the signaling pattern/the output of the signaling network.

By using ordinary differential equations approach, I have designed a simplistic mathematical model which performs basic signaling tasks similar to the signaling process of living cells. I have used a simple dynamical model of signaling networks of interacting proteins and their complexes and studied the evolution of signaling networks described by mass-action kinetics. For the optimization of purpose, an evolutionary algorithm has been used. During the optimization process, the fitness of the networks is determined by the number of signals detected out of a series of signals with varying strength. The mutations include changes in the reaction rate (kinetic parameter), concentration of the signaling molecules, and network topology.

I found that stronger interactions and addition of new nodes lead to improved evolved responses. The strength of the signal does not play any role in determining the response type. The kinetics of the response is predominantly controlled by the concentration levels of proteins. The some of the cross-talks (not all the cross-talks) may also force the cellular response to be transient. This model will help to understand the dynamic behavior of the proteins involved in signaling pathways. It will also help to understand the robustness of the kinetics of the output response upon changes in the rate of reactions and the topology of the network.

Title: Mathematical Modeling and Evolution of Signal Transduction Pathways and Networks

ZUSAMMENFASSUNG

Äußere Reize, die auf Zellen wirken, lösen häufig den Prozess der Signaltransduktion aus, wodurch das Verhalten und die Funktion der Zelle verändert und gesteuert wird. Wenn Rezeptoren ihr entsprechendes Signal registrieren, aktivieren sie eine Vielzahl von Biomolekülen, die im allgemeinen die Signale bis zum Zellkern weiterleiten.

Wie genau Zellen miteinander kommunizieren, z.B. während der Entwicklung eines Organismus oder in einem Krankheitsstadium, stellt immer noch eine große Herausforderung in der Biologie dar. Damit die Zellen eines mehrzelligen Organismus wissen ob sie proliferieren, migrieren, differenzieren oder sterben sollen, nehmen die Zellen aus ihrer Umgebung Hormone und andere Signalmoleküle wahr. Damit diese Signale von der Zelloberfläche an ihrem Wirkort weitergeleitet werden können, transportieren die Signalmoleküle die Information in Form verschiedener chemischer und physikalischer Änderungen, so zum Beispiel Konformations- oder Strukturveränderungen oder durch Aufbau von Komplexen.

Das Hauptziel meiner Dissertation war die Entwicklung eines theoretischen Ansatzes, der dazu dienen soll das Verständnis der Signaltransduktion in biochemischen Netzwerken besser zu verstehen. Der Fokus lag hierbei auf dem Einfluss der kinetischen Parameter, der Netzwerktopologie sowie der Konzentration von Signalmolekülen. Da, wie bereits erwähnt, Zellen in der Regel mehreren Signalen gleichzeitig ausgesetzt sind, bestand eine zusätzliche Aufgabe in der Analyse des Cross-talks in einem Netzwerk beim gleichzeitigen Empfang von zwei verschiedenen Signalen.

Ich habe ein vereinfachtes mathematisches Modell auf der Basis gewöhnlicher Differentialgleichungen entwickelt. Das dynamische Modell eines Signalnetzwerkes beschreibt per Massenwirkungsgesetz die Interaktion von bis zu vier Proteinen mit je drei Zuständen: unmodifiziert und zwei chemische Modifikationen. Weiterhin werden binäre Komplexe explizit einbezogen. Hierbei wird eine zufällige Auswahl von Bindungspartnern aus allen möglichen Kombinationen der Proteinzustände getroffen, um so verschiedene Netzwerk-Topologien zu generieren. In jedem Komplex bekommt ein beteiligter Proteinzustand eine enzymatische Funktion zugeordnet, der bei Dissoziation des Komplexes den Zustand des Interaktionspartners ändert. Zum Beispiel ist das Anhängen eine Phosphat-Gruppe (Phosphorylierung) an Proteine eine häufiger Vorgang bei der Signaltransduktion. Die drei Molekülzustände können dann als unphosphoryliertes Protein bzw. Einfach- und Doppelphosphorylierung interpretiert werden, während die Enzymatische Funktion einer Kinase bzw. Phosphatase entspricht. Die auf diese Weise erzeugten Netzwerke werden einem

Evolutionsalgorithmus unterworfen. Der Grundgedanke ist, dass jedes Netzwerk eine Antwort für einen Satz von Signalen verschiedener Stärke liefern muss. Die Antwort wird dabei so einfach wie möglich definiert. Konkret wurde manuell ein Proteinzustand als Output gewählt, so dass die Konzentration dieses Zustandes bei Vorliegen eines Signals eine Schwelle für eine beliebige Zeit überschreitet. Dadurch wird keine Kinetik fixiert, so dass zum Beispiel anhaltende, oszillierende, adaptive oder transiente Aktivierung des Netzwerkes erlaubt sind. Das Signal selbst ist dabei entweder konstant vorhanden oder liegt als Puls vor. Die Fitnessfunktion ist so konstruiert, dass für jedes erfolgreich detektiertes Signal sich die Fitness um 1 erhöht. Maximale Fitness eines Netzwerkes ist dann gleichbedeutend mit der Detektion aller Signal (unter der Nebenbedingung, dass in Abwesenheit des Signals, das Netzwerk inaktiv bleibt).

Um den Einfluss verschiedener Parameter auf die Funktion von Signalnetzwerken zu analysieren, habe ich vier Grund-Szenarien angesetzt: (i) Die kinetischen Parameter der Protein-Protein-Wechslewirkung werden „mutiert“, analog wie der Austausch einer Aminosäure in Bindungs-relevanten Sequenzen die Affinitäten von Proteinen zueinander oder die enzymatische Aktivität beeinflußt. (ii) Die Topologie des Netzwerkes wird mutiert, indem ein neues Protein mit zufällig gewähltem Satz an Interaktionspartnern bzw. enzymatischer Funktion hinzugefügt wird. Hierbei werden die kinetischen Parameter beim Zufügen des Proteins fixiert und im Laufe der Evolution nicht mehr verändert. (iii) Analog zum Fall (i) werden die kinetische Parameter mutiert. Zusätzlich wird jedoch die Gesamtkonzentration der beteiligten Proteine mutiert. Diese Situation entspricht dem Effekt von stillen Mutationen der kodierenden DNA-Sequenz, die die Effizienz der Proteintranslation oder die Regulation durch micro-RNA ändern und schlussendlich die Proteinexpression beeinflussen kann. Ebenso werden hier die Wirkungen von Mutationen/Regulationen von Transkriptionsfaktoren vereinfacht erfasst. (iv) Zwei Netzwerke mit manuell fixierter aber verschiedener Topologie werden durch eine gemeinsame Interaktion aneinander gekoppelt. Beide Netzwerke müssen jeweils „ihr“ Signal detektieren. Der Einfluss der Kopplung/des Cross-talks wird untersucht, indem die kinetischen Parameter im Evolutionsalgorithmus mutiert werden. Der Evolutionsalgorithmus selbst benutzt für alle Szenarien „elite selection“, dass heißt die ggf. mutierten Nachkommen des besten Viertels (=höchste Fitness) der Netzwerke stellt die nachfolgende Generation. Deren Fitness wird reevaluiert, indem die Antwort auf den Satz verschiedener Signal ausgewertet wird. Dieser Vorgang wird wiederholt, bis die Fitness und das Mittel der mutierten Faktoren sich nicht mehr ändern. Die erste Generation wird hierbei mit Klonen einer zufällig gewählten Topologie angefüllt. Der Endzustand ist eine Generation von Netzwerken, die die Fitnessfunktion optimal lösen (und mit Ausnahme von Szenario ii eine identische Topologie haben). Für jedes Szenario wurde eine Reihe von Wiederholungen simuliert, die alle mit verschiedenen Zufallszahlen gestartet worden sind, so dass

sowohl die Mutationen als auch die Topologie und kinetischen Startparameter der Anfangs-Netzwerke verschieden sind.

Für das Szenario (i) ergaben sich zwei interessante Resultate. Zum einen ist die generische Antwort des Netzwerkes eine anhaltende Aktivierung des Output-Knotens; andere Kinetiken traten praktisch nicht auf bzw. wurden nach kurzer Zeit wieder ausselektiert. Zum anderen erreicht die Fitness der Netzwerke innerhalb weniger Generationen maximale Werte, wobei die kinetischen Parameter eine Drift aufzeigen, die noch viele Generationen anhält. Weiterhin habe ich drei Unterfälle des Szenarios analysiert, in denen die maximale Stärke der kinetischen Parameter unterschiedlich limitiert waren. Hierbei stellte sich heraus, dass es eine Schwelle gibt unterhalb der, die Netzwerke nicht in der Lage sind maximale Fitness zu erreichen. Sobald diese Schwelle überschritten wird, zeigen die Netzwerke die anhaltende Aktivierungskinetik und die kinetischen Parameter driften zu hohen Werten (ohne jedoch das gesetzte Limit zu erreichen). Zu bemerken ist hierbei, dass unabhängig von der Eingangssignalstärke, die Antwort praktisch gleich stark ausfällt ohne notwendigerweise maximal zu sein. So zeigen viele Netzwerke eine 90%-ige Aktivierung des Output während andere für alle Signale z.B. eine 30%-ige Aktivierung aufweisen.

Überraschend ist Szenario (ii) vollkommen analog zu Szenario (i). Je nach gesetztem Limit für die kinetischen Parameter, wenn das neue Protein eingebunden wird, evolvieren die Netzwerke zu maximaler Sensitivität oder versagen bei der Detektion schwächerer Signale. Vermutlich genügt ein Untermenge von Interaktionen mit großen kinetischen Parametern, um die beobachteten Effekt zu erzielen. Daraus habe ich geschlossen, dass die Anwesenheit starker Interaktionen wichtig ist für die anhaltende Aktivierung des Netzwerkes, jedoch die genaue Topologie starker Interaktionen ebenso wie die Netzwerktopologie im allgemeinen nicht bestimmend für das Netzwerk-Verhalten sind.

Dieser Schluss wurde durch die Ergebnisse mit veränderten Protein-Konzentrationen (Szenario iii) unterstrichen. Es zeigte sich, dass unter diesen Bedingungen eine signifikante Menge von Netzwerken eine transiente Antwort auf anhaltend vorliegende Signal erzeugt. Zwischenzeitlich treten sogar Netzwerke auf, die je nach Signalstärke mit transienten bzw. anhaltenden Aktivierungskinetiken reagieren. Diese Netzwerke erwiesen sich jedoch nicht als evolutionär stabil, dass heißt sie sind in der finalen Generation nicht zu finden. Interessanterweise haben Änderungen der Konzentration des Proteins, dessen Aktivierungszustand als Output gewählt wurde nur minimalen Einfluss auf die Kinetik des Netzwerkes.

Die Analyse des Cross-talks (Szenario iv) ergab, dass nur einige bestimmte Kopplungen nicht-triviale Ergebnisse aufzeigen. Ein häufiger Fall ist hierbei, dass die Ko-Aktivierung beider Signale zu einem

transienten Verhalten eines der beiden gekoppelten Netzwerke führt. Andere Kopplung erzeugen hingegen nur eine vollständige Inhibierung oder haben keine sichtlichen Einfluss der Signale/Netzwerke aufeinander.

Kritisch anzumerken ist jedoch, dass die Ergebnisse an die Wahl der Fitnessfunktion gekoppelt sind. Eine andere Fitnessfunktion, die z.B. eine oszillierende Lösung einfordert, kann eventuell eine stärkere Sensitivität gegenüber der Topologie oder den kinetischen Parametern des Netzwerkes aufweisen.

Zusammengefasst zeigen meine Ergebnisse, dass sowohl die Interaktionsstärke als auch die Netzwerktopologie gegenüber den relativen Konzentrationen der beteiligten Moleküle eine geringe Rolle bei der Festlegung der Kinetik des Netzwerkes spielen. Diese Erkenntnis erscheint überraschend positiv für die experimentelle Untersuchung von Signaltransduktion. Sowohl die (vollständige) Netzwerktopologie als auch die zugehörigen kinetischen Parameter sind schwer zu erfassende Parameter, insbesondere da die Identifkation von Signal-Subnetzwerken eher experimentellen Einschränkungen, denn einer umfassenden Analyse einer Zelle unterworfen ist. Demgegenüber erscheint eine Bestimmung der Gesamtkonzentration von Proteinen eine vergleichsweise einfach zu lösende Aufgabe, die im Hinblick auf meine Ergebnisse eher geeignet scheint, das Signalverhalten für ein konkretes Problem zu bestimmen bzw. im Umkehrschluss durch biochemische oder genetische Eingriffe zu modulieren.

Chapter 1 INTRODUCTION

1.1 Signal transduction process

Signal transduction is a major step in inter- and intra-cellular communication [1]. In signal transduction processes, an external stimulus is transformed into a cellular response through a network of proteins that ultimately alters the behavior of the cell[2,3]. There are many diseases which arise due to the malfunctioning of signal transduction pathways such as cancer, diabetes, and anchondroplasia (dwarfism) which are the diseases caused due to malfunction in signal transduction pathways [4].

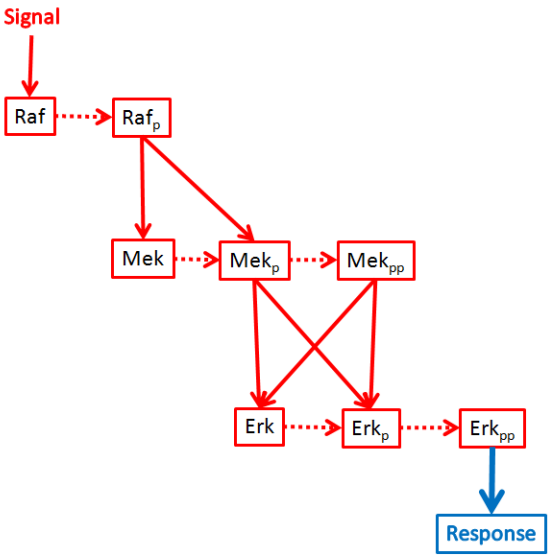

Figure 1.1MAP kinase cascade. After receiving the signal from RasGTP (active Ras), inactive Raf is phosphorylated (activated) and phosphorylated Raf activates Mek and active Mek further activates inactive Erk to active Erk. (Raf_p - active Raf, Mek_p - single phosphorylated Mek, Mek_{pp} - double phosphorylated Mek, Erk_p - single phosphorylated Erk, and Erk_{pp} - double phosphorylated Erk).

To study how in exact the cells communicate is a challenging task in all aspects of biological science, from developmental stage to diseased state. In multicellular organisms, cells detect the presence of neighboring cells, hormones, and other biomolecules before and during making a decision such as whether to proliferate, migrate, differentiate, or to die. In order to transduce the information from one level to another level, the SMs involved in signal transduction can go to different kind of physical changes such as conformational changes, structural modifications, formation of scaffolds etc. The most common example is the posttranslational modifications of proteins (Figure 1.1), e.g., phosphorylation and dephosphorylation [5].

For a more detailed understanding about the transmission of signal from one molecule to another, I take MAPK pathway as an example that shows different response types depending on the cell type and/or stimulus (Figure 1.1). This pathway involves Raf, MEK (MAPK/ERK kinase), and ERK (extracellular signal regulated kinase) and is considered to be centrally involved in cellular decision making processes where small quantitative differences often lead to major phenotypic changes [6]. It has been shown that the upstream molecules induce quantitative and qualitative differences in the duration and magnitude of ERK activity that regulate the function and behavior of a cell. The MAPK pathway is a prototype for the general scheme of signal transduction, in which, after receiving a signal from ligand-bound receptors, the involved proteins are altered (''activated'') by posttranslational modifications[5,7]. Subsequently, the active form activates the other inactive proteins by means such as recruitment to specific locations, altering the enzymatic activity, or conformational changes exposing binding sites for further binding partners.

The aim of my thesis was to focus mainly on the intracellular signal transduction at the molecular level and to develop mathematical models which can be applied to investigate the signal transduction processes in T-cell signaling. Before introducing my work, first of all I would like to introduce the biological signaling process in T-cell activation and then a specific signal transduction pathway (mitogen activated protein kinase (MAPK) pathway) to illustrate the information flow from the receptor level to the effector level (Figure 1.1).

1.2 T-cell signaling

T-Cell Receptor (TCR) activation leads to the activation of several biomolecules resulting in a number of signaling cascades (Figure 1.2). The dynamics of these cascades ultimately determine cell fate through the regulation of cytokine production, migration, cell survival, proliferation, apoptosis, and differentiation. In TCR activation, in the initial step after detection of appropriate ligand, phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) takes place on the cytosolic part of the TCR/CD3 complex by lymphocyte protein-tyrosine kinase (Lck). Where it becomes activated, promotes the recruitment and phosphorylation of downstream signaling molecules. Zap-70 phosphorylates SLP-76 the phosphorylated SLP-76 promotes the recruitment of Vav (a guanine nucleotide exchange factor), the adaptor protein GADS and inducible T cell kinase (ITK). ITK phosphorylates PLCγ1 (phospholipase C γ1) which results in the hydrolysis of PIP2 (phosphatidylinositol 4,5-bisphosphate). The hydrolysis of PIP2 produces the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG activates protein kinase C theta (PKCθ)and the MAPK pathway which also promotes nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation. IP_3 triggers the release of Ca^{2+} from storage organelles such as endoplasmic reticulum (ER), mobilizes the Ca^{2+} , and helps in the entry of extracellular Ca^{2+} into the cells through calcium release-activated Ca^{2+} (CRAC) channels [8]. CD28 recruits PI3K (phosphatidylinositide 3-kinase). PI3K produce PIP3 and PIP2. PIP3 recruits PDK1 (phosphoinositide-dependent kinase-1) and protein kinase B (PKB also called AKT) which leads to the phosphorylation of AKT by PDK1. Finally, this phosphorylated AKT can activate the downstream signaling molecule ERK (MAPKKK). Ca^{2+} binds to calmodulin which binds to calcineurin which leads to the activation of calcineurin. This active calcineurin finally activates NFAT (nuclear factor of activated T cells) [9]. From previous studies it is known that the temporal behavior of Ca^{2+}, MAPK, and NF-kB determines the cellular decision [6,10,11].

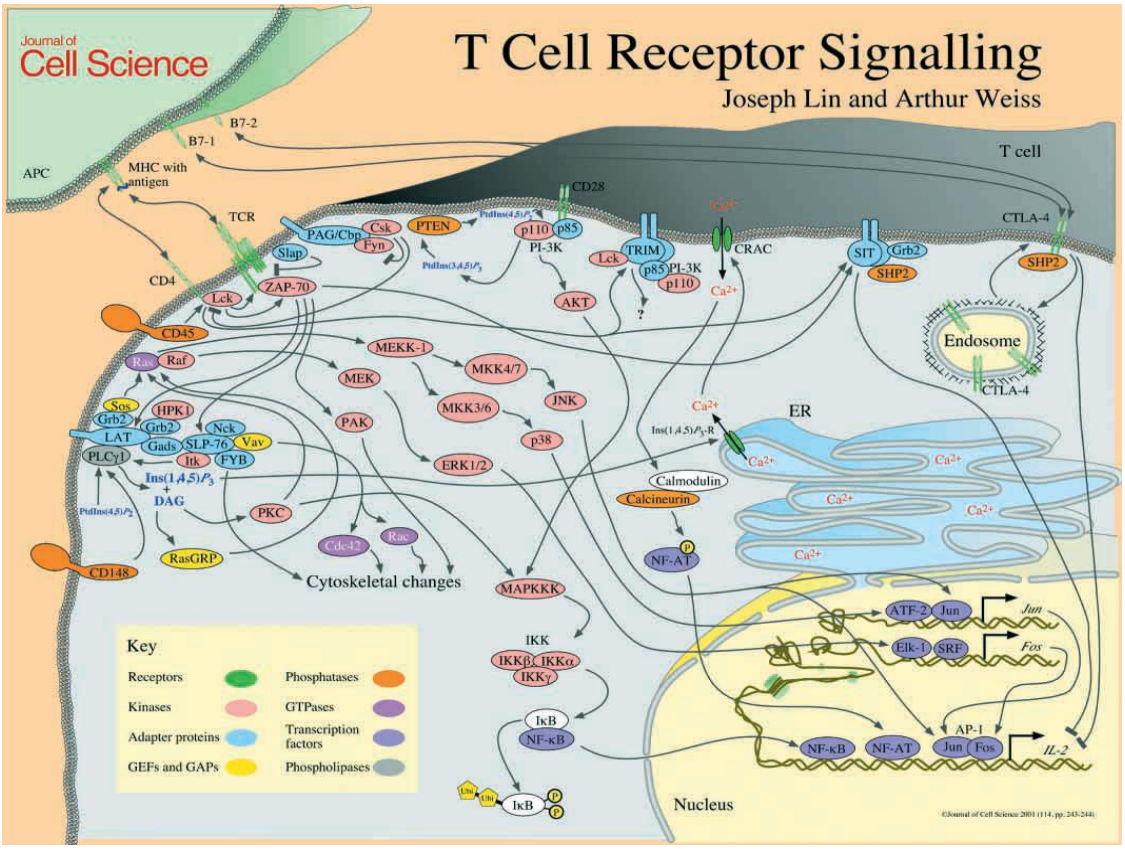

Figure 1.2 T-cell signaling: In T-cell signaling TCR triggering leads to the activation of several signaling molecules and feeds a number of signaling cascades which determine cell fate decision (Figure adapted from Lin J. and Weiss A. 2001[8]).

1.3 Role of input signals, interaction strength, addition of new protein, concentration levels on the cellular response

From previous studies it is known that cells can communicate through the processing of information from one level to another by controlling the temporal behavior (dynamics) of the signaling molecules. The temporal dynamics of these signaling molecules control the cellular decision (such as apoptosis, proliferation, or differentiation, etc.). The dynamics is used to describe the change in the concentration, activity, modification state, or localization of the signaling molecules over time [12]. The signaling mode encodes the information in the frequency, amplitude, duration, or the other features of the temporal signals.

In previous studies, various modeling approaches have been applied to investigate the behavior of signaling networks (SNs). Francois and Hakim (2003)[13] developed a model to generate genetic circuits which can deliver a variety of functional behaviors and demonstrated the vital role of post-transcriptional interactions, i.e. protein-protein interactions for controlling gene regulation. They used an evolutionary approach. This evolutionary approach has been extended by the others to protein-protein interaction networks with specific functional characteristics: oscillators, bistable switches, homeostatic systems, and frequency filters [14,15].

In a previous study [16], the role of kinetic parameters, local concentration variations, and feedback loops have been studies. In this work, they have presented a computational model of the GTPase-cycle module that predicts the interplay of local G protein, coupled receptor, and GTPase-activating protein (GAP) concentrations which gives rise to different regimes and numerous intermediate signaling phenomena. This model provides mechanistic insights into the regimes under which distinct GTPase-cycle modules function and yield a wide range of biochemical phenotypes and provides the quantitative framework for the experimental investigations of GTPase-cycle modules.

To predict the function of a signaling module it is necessary to understand the design principle of SNs that underlie the behavior, function, and robustness. From experiments neither the topology of a SN nor the kinetic parameters of its underlying elementary interactions are known in detail such that it remains open how sensitive the function of a network is due to these parameters. In addition to it, it is also hard to measure the concentration levels of all the signaling molecules involved in transmitting the signals from one signaling levels to another (like receptor level, mediator level, and effector

level as well as post-translational modification thereof). Therefore, it seems appealing to investigate the roles of change in SN topology, kinetic parameters, and the concentration levels of signaling molecules to get a glimpse of how important each detail is for the outcome.

1.4 Cross-talk between signal transduction pathways

From previous work it is known that the receptors for GFs (growth factors) and the ECM (extracellular matrix) are ubiquitously expressed in multicellular organisms. For signal transduction process, integrin-type ECM receptors anchor cells to their surrounding and concomitantly activate intracellular signaling pathways. For this signal processing system it has been shown that there are different possible cross-talk (Figure 1.3). In case of activation of cellular signaling pathways, the interaction between integrin and GFRs (growth factor receptors) can take place in different ways: (i) The downstream action of the two receptors can take place concomitantly and independently (concomitant signaling), (ii) integrins can also gather different proteins around themselves and help GF-dependent GFR signaling (collaborative signaling), (iii) even in the absence of GFs, integrins can also directly activate GFRs (direct signaling), and (iv) as it is also known that GFR-generated signals lead to the activation of integrin gene expression, so the increased level of integrins on the cell surface can also further activate signaling by GFRs (amplification of signaling) [17].

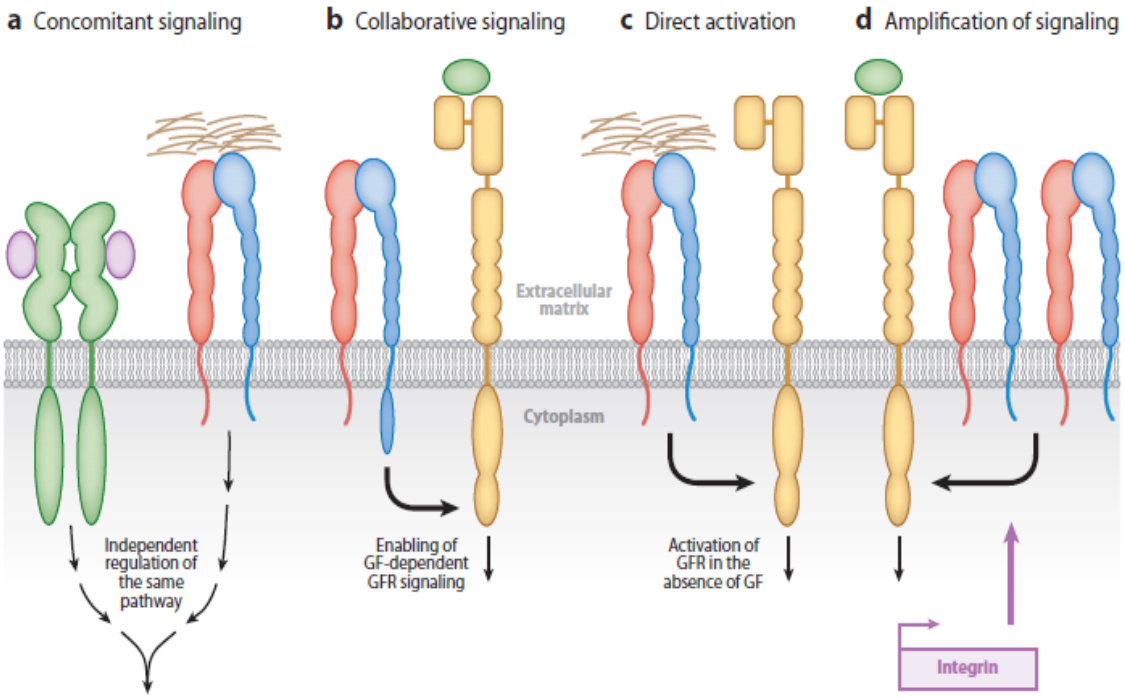

Figure 1.3 Possible interactions between integrin and GFRs. (a) Concomitant signaling: interaction between integrin and GFRs are concomitant and independent, (b) Collaborative signaling: Here, integrin brings more molecules close to itself and help in GF-dependent GFR signaling, (c) direct activation: in this case integrin can directly activate GFRs even in the absence of GFs, and (d) Amplification of signaling: GFR-generated signals can lead to the activation of integrin gene expression and this increased integrin gene expression may further activate GFR signaling (Figure adapted from Ivaska J. and Heino J., 2011[17]).

In addition to the above mentioned work on cross-talk, there are some other interesting facts about the role of cross-talk on the cellular response revealed by a theoretical approach [18]. Here, it has been shown thatin a single cell, during signal transduction process, the cell responds to only one of the stimuli even when exposed to both. They also claim that these pathways achieve specificity by filtering out spurious cross-talk through mutual inhibition and the variability between the cells allows for heterogeneity of the decisions (Figure 1.4). Although components of a signaling system shared between pathways provide the signaling network with a capacity for signal integration. The problem to the study mentioned above addresses is: signals transmitted through one pathway could cross-activate the other through these shared components, leading to a loss of specificity. In order to overcome this problem, the signaling network must be able to respond properly to the external stimuli. For this purpose they proposed two fundamentally different mechanisms allow signaling pathways that share components to respond specifically to any one stimulus.

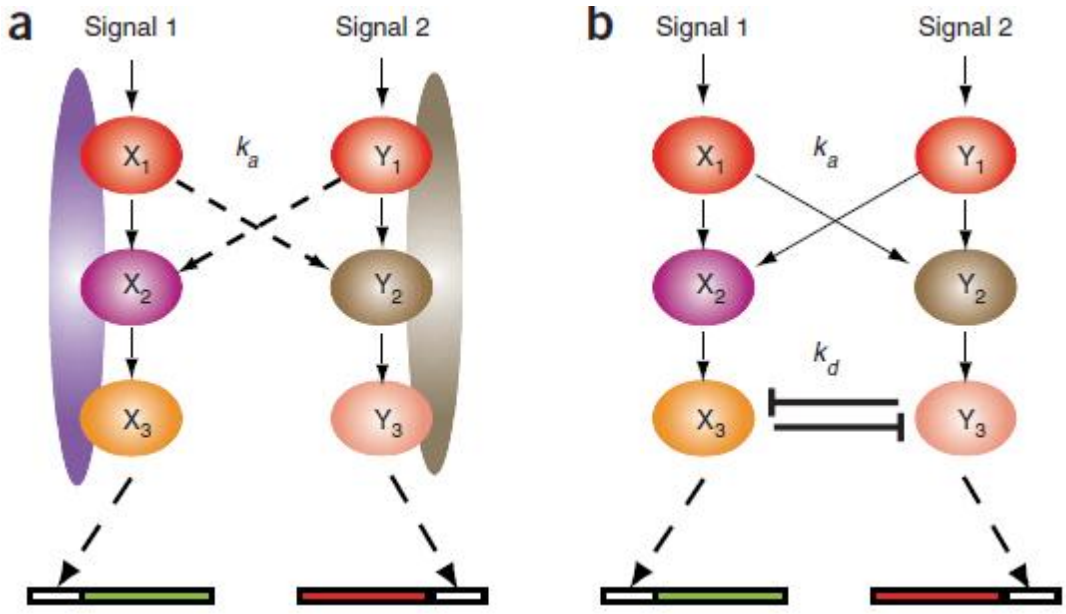

Figure 1.4 Different mechanisms for achieving specificity of two parallel signaling pathways yield different responses after exposure to both signals. (a) Scaffolding proteins insulate two pathways from each other. For perfect insulation, the rate of cross-activation ka equals 0. When exposed to both signals simultaneously, both pathways are active. (b) Two pathways show cross-activation but maintain specificity by cross-inhibition of X3 and Y3 (adapted from McClean M N. et al, 2007[18]).

The first mechanism is insulation. This can be achieved by incorporating the shared component into distinct macromolecular complexes— one for each signal to be processed (Figure 1.4a). The second mechanism is mutual inhibition, which is used to eliminate unwanted interactions between the pathways (Figure 1.4b). Through mathematical modeling, they show that they can use physiological measurements to distinguish between these two mechanisms of achieving specificity. They then apply their analysis to the specific example of MAPK pathways in the yeast S. cerevisiae [18].

1.5 Evolutionary Algorithm

Evolutionary algorithm is a type of evolutionary computation which is mainly inspired by biological evolution concepts such as reproduction, mutation, and selection[19]. In the past evolutionary algorithm have been applied to address many biological problems and has been found as a helpful approach[20]. This algorithm is based on initial population, mutation, selection, and iteration[19]. During optimization, the fitness function plays important role in determining and selecting most appropriate solution. In general, heritable variation requires some kind of change in the features and/or parameters used for the preparing the initial populations. These changes are repeated again and again and each iteration is as one generation. Before entering into iteration cycle, selection process is applied. Based on the fitness the set of a population is declared as the fittest population. In each iteration, the sets of the population are created (similar to biological term reproduction). There are different known selection process e.g., elite selection (I have used in the thesis work). The more details of this algorithm, I have shown in the method chapter.

1.6 Aims and objectives of my Ph.D. thesis work

As I have mentioned in the previous section 1.3 that the temporal dynamics of the signaling molecules play critical roles in controlling the cellular behavior. Thus, a good understanding of the time-scale of a particular system is crucial for determining and understanding the cellular behavior. Where the system will help to understand the factors such as the input signals, kinetic parameters, change in signaling network topology, the concentrations of the signaling molecules, motifs and domains of signaling proteins responsible to control and affect the temporal behavior of the signaling molecules. In addition to it, the system will also help to understand the roles of different possible cross-talk of the signal transduction pathways and the network motifs on the cellular function.

In my Ph.D. thesis work, I have addressed mainly the following problems:

- **Effect of kinetic parameters of the reactions, input signals, concentration of SMs (signaling molecules) on the final response:**

  As the problem mentioned in the previous section that the temporal dynamics of the signaling molecules control the cellular decision, so it sounds to be interesting and promising to investigate the roles of the kinetic parameters (in experiment, it is hard to measure the kinetic parameters) and input signals on the temporal dynamics of the signaling molecules.

- **Cross-talk of signaling pathways**

  In section 1.5, I have introduced that many diseases such as cancer, diabetes, obesity, and asthma are caused due to defects in multiple genes and pathways. And it is also known that the genes to be functional, they need to interact with many more molecules. Since, in the above mentioned diseases, it is not that one gene is responsible but there are many other molecules involved. Which leads the current one-target-one-compound approach in drug discovery and development to failure to deliver as many efficacious medicines as expected in the post-genomic era. So, instead of investigating on a particular pathway, it may be a positive step to investigate also the possible cross-talk between the signal transduction pathways and their impact on the output response.

There are many important works which have been published related to these works but the main feature of our work is to develop and study the cellular behaviors due to the perturbation in the interaction. This approach will bring our theoretical predictions very close to the experimental predictions and will also help the experimentalists to save the time and manpower and will guide to proper directions for performing new experiments.

Chapter 2 RESULTS

Before discussing the results, I would like to present an overview of the ten different subsections. These ten subsections are organized as:

- Subsection 2.1 - In this subsection, the evolution pattern of SNs has been discussed for the system where the SNs have been evolved by allowing the change in the kinetic parameters only. The concentration of different SMs are fixed and equal.
- Subsection 2.2 - In this subsection, the kinetics of the output response and the evolution pattern of kinetic parameter (interaction strength) for the evolved networks have been shown for the system where the SNs have been evolved by allowing the change in the kinetic parameters only. The concentration of different SMs are fixed and equal.
- Subsection 2.3 - In this subsection, the evolution pattern and the kinetics of the output response of SNs has been discussed for the system where the SNs have been evolved by allowing the change in the kinetic parameters only. The concentration of different SMs are fixed and unequal.
- Subsection 2.4 - In this subsection, the evolution pattern and the kinetics of the output response of SNs has been discussed for the system where the SNs have been evolved by allowing the change in the kinetic parameters and also in the concentration of different SMs during the evolutionary process.
- Subsection 2.5 -To verify my theoretical finding, one of my collaboration group member (Dr. Tina M. Schnöder) has performed western blot analysis for MEK and ERK molecule of MAPK pathway. This western blot result have been shown here.
- Subsection 2.6 -Here, I have discussed the signal-response relationship for all the different conditions used for simulation.
- Subsection 2.7 - In order to check the effect of removal of input signal after certain time, I have run the simulation where the input signal remains present only for fixed time points. This result for the removal input signal has been shown here.
- Subsection 2.8 - This subsection contains the kinetics of the partially active (single phosphorylated) SMs.
- Subsection 2.9 - Explains the addition of new nodes in the minimal model and its evolution.

- Subsection 2.10 - Finally, I have investigated the feedforward, feedback and cross-talkpresence during signal transduction process.

For this work, I have set up a simplified model to represent a signal transduction pathway allowing two post-translational modifications of similar to the MAPK cascade [21]. In order to transduce the signal, I have included protein-protein interactions, protein phosphorylation and dephosphorylation [22]. Double phosphorylated proteins act as fully activated and single phosphorylated molecules as partially activated molecules. Note, that the term phosphorylation is used for convenience as any other post-translation modification adding a small chemical group, lipid, protein or carbohydrate modifying a protein's spectrum of interaction partners or enzymatic activity are covered by the model (Figure 2.1).

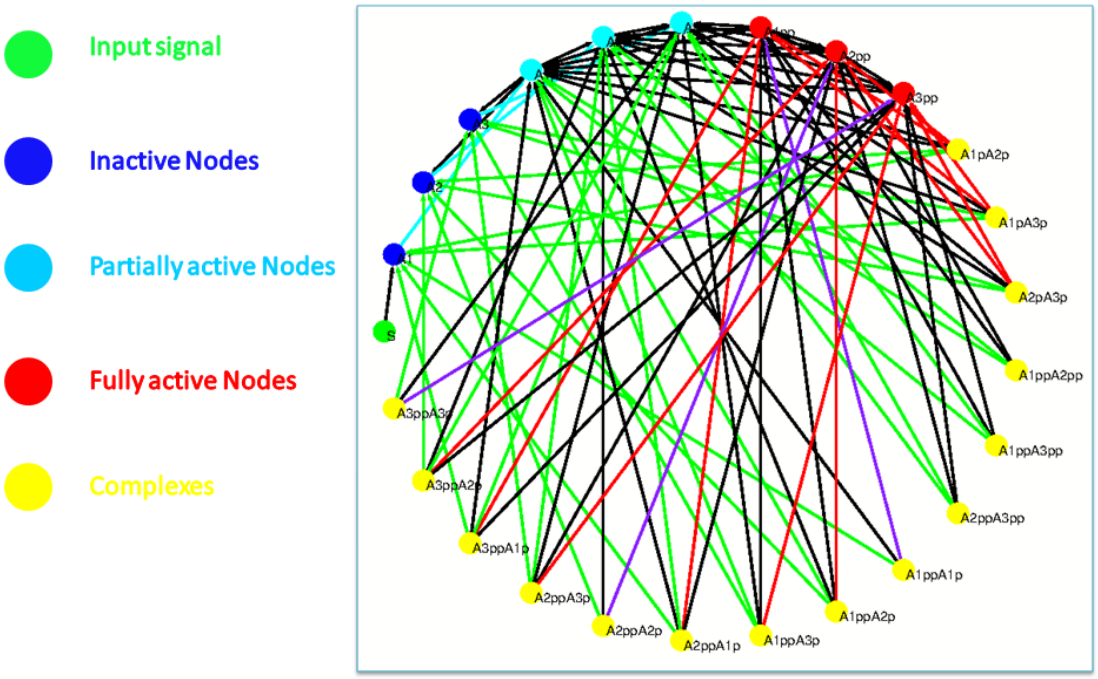

Figure 2. 1STNs with random interactions and bimolecular complexes formation. S represents the input signal (green color node), A1, A2, and A3 denote inactive signaling proteins (blue color nodes), their partially active (single phosphorylated (cyan color nodes)) forms are A1p, A2p, and A3p, respectively. The fully active forms (red color nodes) are A1pp, A2pp, and A3pp. And all other nodes (yellow color) represent the possible complexes formed during signal processing.

The interaction between the signaling proteins are set up randomly to create the initial population as well as when adding proteins during evolution. In my current model, I have not classified the proteins of the SN.

Once the receptor receives the signal then it can activate other signaling molecules. All the signaling molecules are allowed to phosphorylate or dephosphorylate each other (Figure 2.1) and the final products will be formed depending on the complex. All the reactions in this model are bimolecular, autophosphorylation and homodimer formation are not allowed. Every molecule that becomes partially (single phosphorylated) or full active (double phosphorylated) can interact with any other molecule in any state. These interactions lead to complex formation. The complexes can dissociate without changes to its constituents or upon modifying on of it by means of phosphorylation or dephosphorylation. The interaction of two partially active molecules produces either one of them being fully activated (dual phosphorylated) or inactivated (dephosphorylated) without changing the other reacting partner's state (attributing it an enzymatic role) as shown in Figure 2.1. Which of the possible reactions are realized is determined randomly once at the beginning (with constraints, see next paragraph), thus setting up the network topology.

I start the evolution of SNs by assuming that the basic task of signal transduction is to provide an above-threshold response to a signal which is generated by a ligand binding to its receptor (see Methods). The response is measured at a pre-selected node. When the activation state of this node crosses a threshold the network for an arbitrary amount of time, the SN is considered to be successful in the detection of the signal and the fitness is increased by $F_{factor} = 1$ otherwise the fitness for the signal in question remains 0. For the task to detect multiple signals of different strengths the fitness contributions are summed up. During simulations signals are either present throughout the simulation or given as a pulse of fixed duration. I have investigated the evolution of SNs and their activation pattern underfollowing types of changes: variations of kinetic parameters, addition of new nodes, variation in the concentration of the SMs, and the cross-talk of signal transduction pathways. The evolutionunder all the above mentioned perturbations are investigated independently and compared using replicates with identical parameter settings but different seeds for the pseudo-random number generator.

2.1 Evolution of SNs with varying interaction strength

I have analyzed the effect of strong and weak interactions by simulating SNs evolution for three different regimes: weak (k < 10, dimensionless parameter), moderate (k < 30), and strong interactions (k > 30). When the interaction strength remains below a certain value (k < 10 in the model setting) the networks are unable to reach maximum fitness (Figure 2.2). At the same time the fitness of the population fluctuates significantly. If the interaction strength remains below a value of 30, then the networks population reaches almost maximum fitness, but exhibits a considerable amount of fitness fluctuations. Further increase in the interaction strength (k > 30) suppresses fluctuations in the fitness, i.e., a population has evolved in which virtually all the networks are able to detect every single input signal. For all parameter regimes I have observed that the evolutionary process approaches a steady state after less than 30 generations (Figure 2.2) (data published [23]).

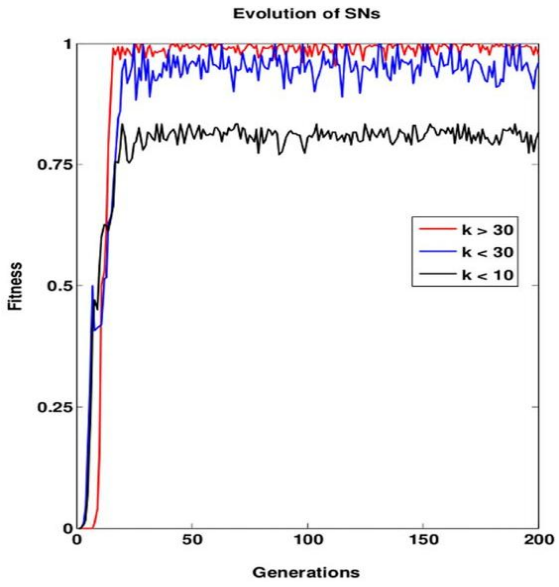

Figure 2. 2 Evolution of SNs by mutating kinetic parameters. The mean fitness of fitness (F_{norm}) during the evolution of SNs for three different regimes of maximal interaction strengths: weak (k < 10), moderate (k < 30), and strong (k > 30) interactions (Number of generations: 200, Number of SNs: 200, Threshold level: $\frac{1}{10}^{th}$ of the initial concentration level of protein).

2.2 Strong interactions promote signal strength-independent and robust activation patterns

In order to understand how the evolved networks manage the task of detecting signals, it is important to analyze the dynamic behavior of the networks over the evolution period. Due to the random generation of the initial kinetic parameters, the activation patterns of the nodes of the networks are different in each starting population. In analogy to the fitness function, I have defined a protein to be strongly active when its relative fraction in the active state passes a given threshold. Any other non-zero value defines the node as weakly active. I observe that the output node initially passes the signaling threshold only for the stronger signals while during the course of evolution the networks detect more and more signals (Figure 2.3). Depending on the strength of interactions between the proteins and their complexes, most successfully evolved networks show a similar activation pattern with little change during the following generations.

When the networks evolve and kinetic parameters are allowed to mutate within the range of 1 to 10 then the activation pattern is weak even with respect to strong input signals. Also the initial variable activation pattern remains throughout the entire evolution period (marked as Sys III in Figure 2.3). Hence, weak interactions do not produce signaling strength-independent activation patterns. From this observation I conclude that the kinetics of the output nodes of the evolved networks are not robust when proteins interact weakly. The same topology, however, can detect signals when the interaction strength is increased. Evolution of networks with kinetic parameter values in the range of 1 to 30 show strong activation and also display a similar activation pattern throughout the SN population after about 50 generations. If networks are evolved permitting even higher kinetic parameter values ($k > 30$) then the networks quickly adapt their dynamics to strong and robust activation patterns. The output node of each of the successful network becomes activated soon after detecting the signal and shows almost equal response strength and similar activation pattern irrespective of the input signal strength (marked as Sys II and I inFigure 2.3) (data published [23]).

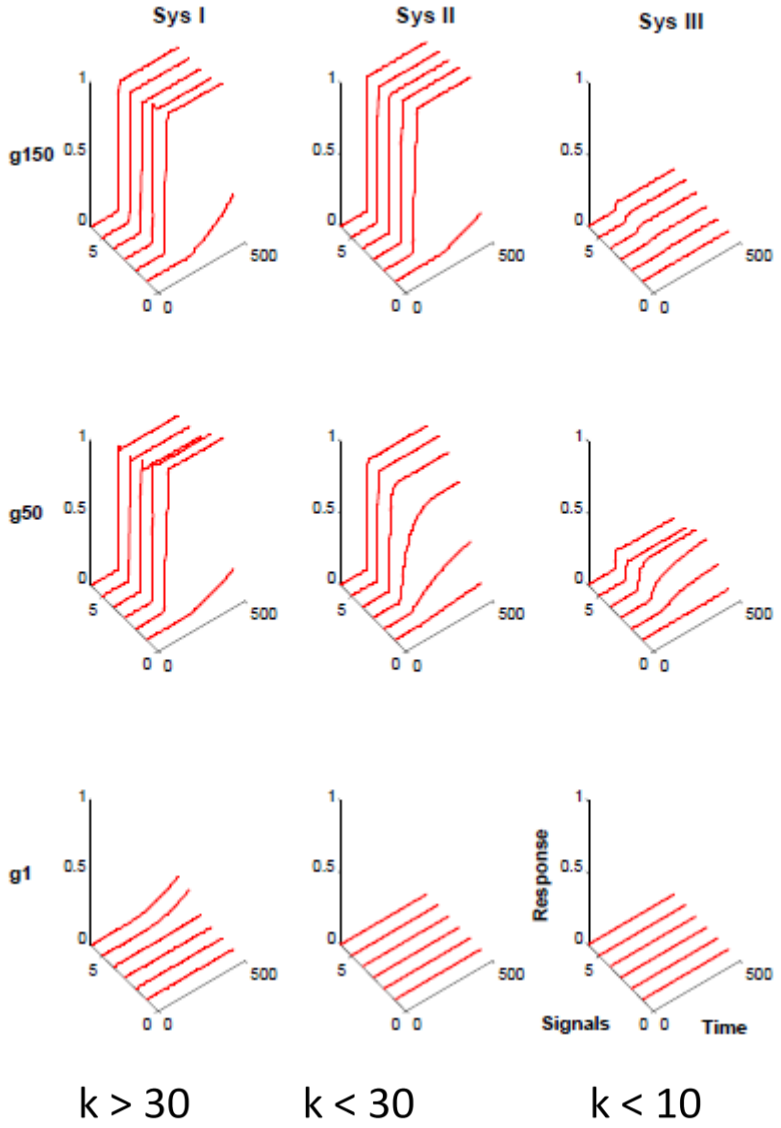

Figure 2. 3 Kinetics of the evolved networks. Shown are the networks with the highest fitness score from one of the simulation runs. g1, g50, and g150 denote the generation 1, 50, and 150, respectively. The six solid lines show the kinetics of activation of the output node in response to six different input signal strengths (strength increases with signal index). Signals are provided at time t~150. Sys III, weak interactions (k<10). Sys II, moderate interactions (k<30). Sys I, strong interactions (k >30).

In these simulations, I observe that about 50 generations are sufficient to achieve a stationary distribution of activation patterns all of which show strong activation provided the interaction strength is sufficiently high (Figure 2.3). Yet, an investigation of the mean kinetic parameter shows that thereis a drift towards stronger interactions in the following generations (Figure 2.4). It takes as long as 150 generations to achieve a stationary population for the regime in which the strongest interactions are permitted (Figure 2.4). A detailed investigation of the parameter distribution of the final generation shows that there is no preferred pattern of kinetic parameters (Figures 2.5 and 2.6). In a brief phase around the time when the population reaches maximal fitness the population is dominated by the networks having rather similar kinetic parameters. Subsequent generations then diversify again resulting in a wide distribution of kinetic parameters used by the networks. This suggests that none of the interactions is critical in the sense that it is subject to a strong selection pressure. Moreover, the same topology can solve the task using very different parameter setups which appear to form a connected set in the parameter space given the chosen fitness function. However, a further increase in the interaction strength does not provide a selective advantage as it has only minimal influence on the activation pattern of the network (data published [23]).

Evolution of kinetic parameters

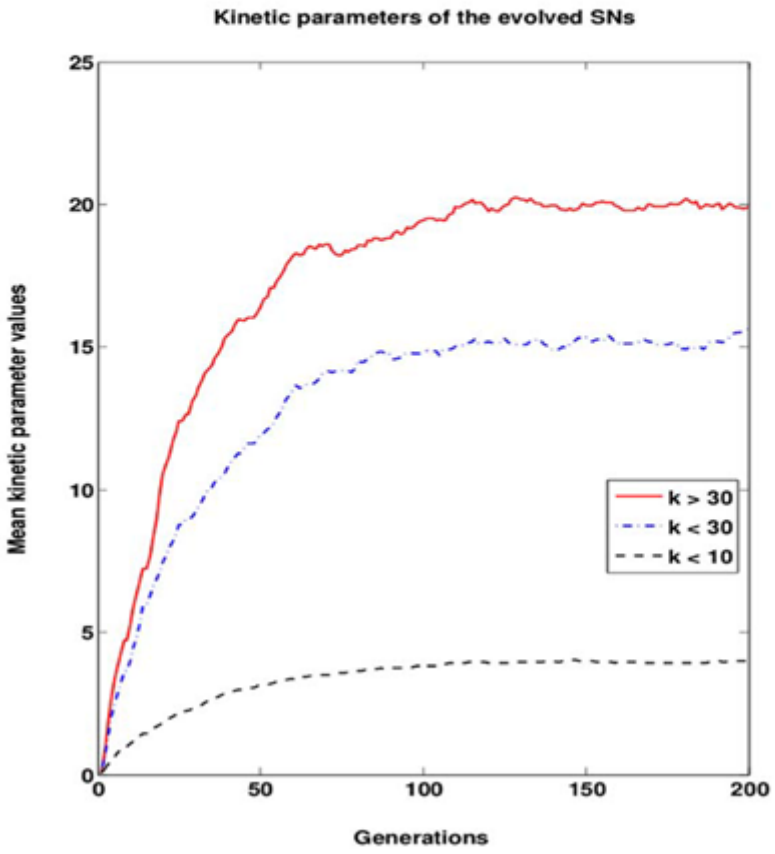

Figure 2. 4 Mean kinetic parameter values of networks during evolution. The numeric values for all kinetic parameters in the SN are averaged omitting the formal difference of first-order and second-order reactions (see Methods).

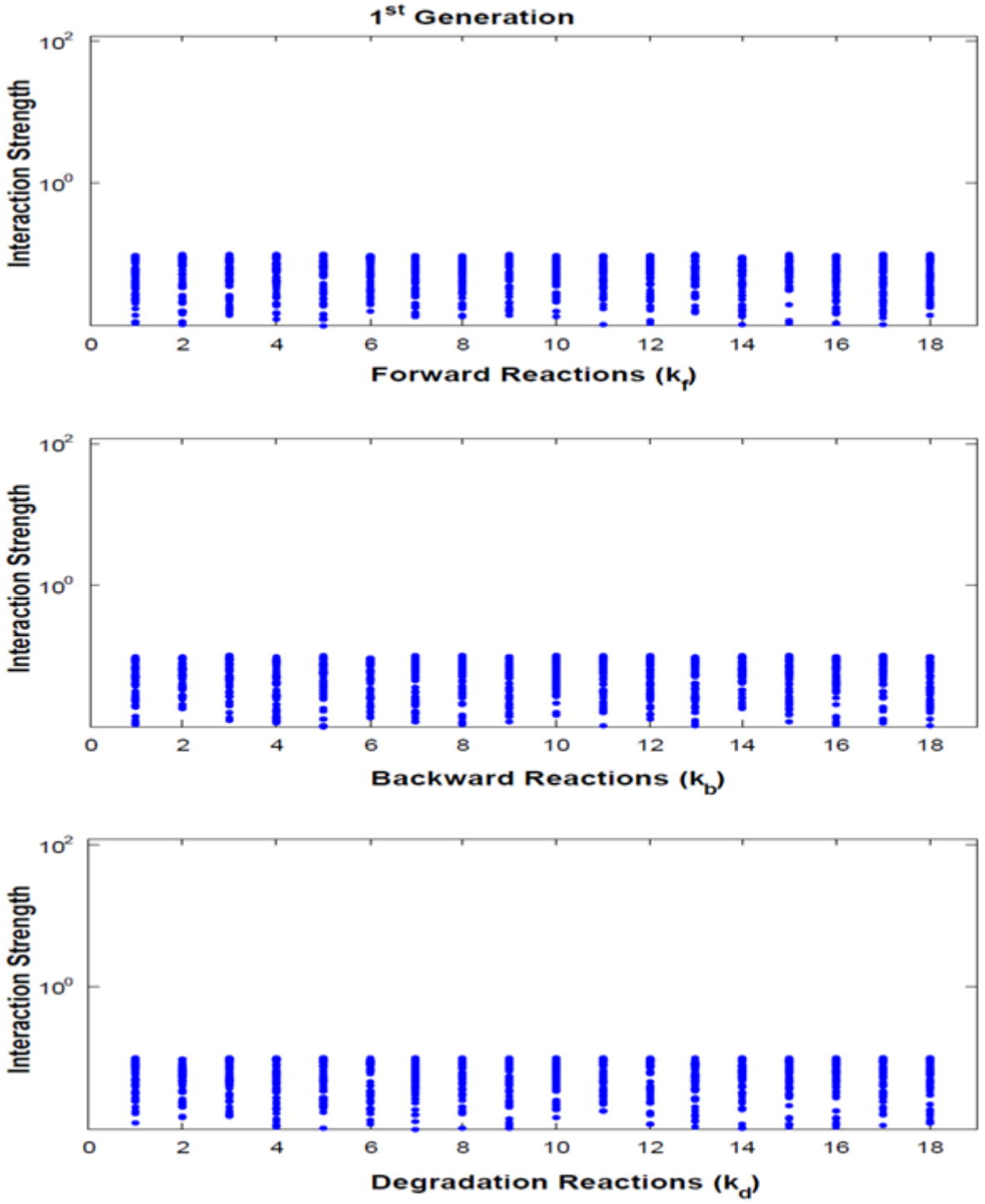

Figure 2. 5 Kinetic parameter distribution of the initial networks (in 1st generation). The numeric values for all kinetic parameters in the SN are averaged omitting the formal difference of first-order and second-order reactions (see Methods).

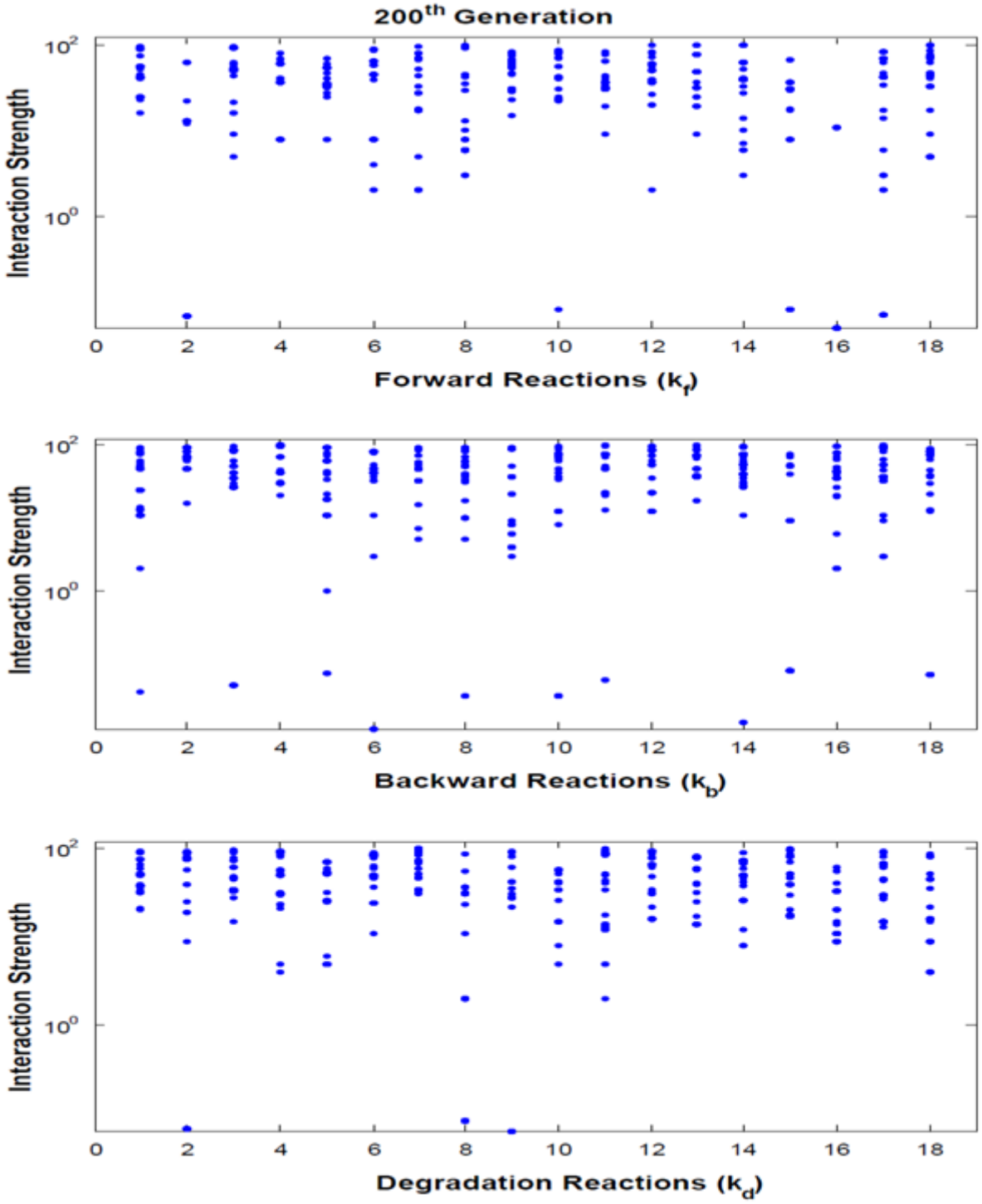

Figure 2. 6 Kinetic parameter distribution in the fully evolved networks (in 200th generation). The numeric values for all kinetic parameters in the SN are averaged omitting the formal difference of first-order and second-order reactions (see Methods).

2.3 Difference in the concentration of the signaling molecules lead to transient activation

To investigate the modulation of cellular response from adapted to non-adapted and vice-versa, I have evolved the networks by applying the evolutionary algorithm (see Methods) under four different conditions. (i) Evolution of STNs due to variations in the kinetic parameters while the concentrations remain fixed. Different from previous section the concentration of the receptor, intermediate, and effector molecules are chosen to be different (Figure 2.7, SystemI). (ii) Evolution of STNs due to variations in the concentration of the receptor molecules and kinetic parameters (Figure 2.8, System II). (iii) Evolution of STNs due to variations in the concentration of the intermediate signaling molecules and kinetic parameters (Figure 2.9, System III). (iv) And evolution of STNs when the concentration of the effector molecules and kinetic parameters is varied (Figure 2.10, System IV). Each system of System I to IV has three different subsystems subsystem a (interaction strength <10), b (interaction strength <30), and c (interaction strength <100). The evolution with these four different conditions are investigated independently and compared using replicates with identical parameter settings but different seeds for the pseudo-random number generator. Then the fitness (F_{norm}) of the networks in each generation is determined (Figure 2.7A, 2.8A, 2.9A, and 2.10A). The fitness shown here represents the mean fitness values of the fitness (F_{norm}) in the respective generations. Only the successfully evolved networks were allowed to enter next generation. The different sets of concentration for System I have been defined in the method section.

I have compared the effect of strong and weak interactions (System I) as well as the consequences of changes in the interaction strengths and concentrations of the signaling molecules during the evolutionary period (System II, III, and IV). In System Ia, when the interaction strength remains below a certain threshold (k <10 in our model setting) the networks are unable to reach maximum fitness (Figure 2.7A). It means the evolved networks do not detect every input signal (Figure 2.7B). Further increase in the interaction strength (k <100) leads the fitness to the maximum (Figure 2.7A), i.e., a population has evolved in which virtually all the networks are able to detect every single input signal (System Ic) (Figure 2.7B). These results demonstrate, that the conclusions made previously with respect to the role of the kinetic parameter strength vs. fitness also hold for different concentrations of the molecules.

In System IIa and IIIa, when the interaction strength remains below a certain threshold (k <10 in our model setting) the networks are unable to reach maximum fitness and at the same time the fitness curve shows significant fluctuation (Figure 2.8A and Figure 2.9A). Based on this evolution pattern, I conclude that the evolved networks do not detect every input signal (Figures 2.8B and 2.9B). Further increase in the interaction strength (k <100) leads the fitness to the maximum and the fitness shows rapid fluctuation even at stronger interactions (Figures 2.8Aand2.9A), i.e., a population has evolved in which virtually all the networks are able to detect every single input signal (System IIb, IIc, IIIb, and IIIc) (Figures 2.8B and 2.9B).

The evolution pattern of STNs in System IV where the mutation in the concentration of the effector molecules in addition to kinetics parameters has been allowed (Figure 2.10A) appears similar to the evolution pattern of the STNs in System I for all the three kinetic parameter regimes (Figure 2.7A). While the kinetics of the evolved networks (Figure 2.10B) are different in System IV than the kinetics of the evolved networks in System I (Figure 2.7B). In system IV, there are significant number of the evolved networks with sustained response while in system I, most of the evolved networks show transient response with few exceptions.

I have run the simulations for STN evolution at constant concentration and with the possibility of variation in the interactions strengths in each generation for three different sets of concentrations of signaling molecules. (A) Simulation with the concentration of the receptor molecule set to 10, intermediate molecule set to 5, and effector molecule set to 1 (System AI). (B) Simulation with the concentrations of the three molecules set to 5, 10, and 1 (System BI). (C) The concentrations of the three molecules set to 1, 5, and 10 (System CI). Each simulation was performed for three different regimes of interaction strength as mentioned in the previous section. I analyzed kinetics of the evolved networks in System AI, BI, and CI (concentrations are fixed but not equal) and observed that the output node shows a transient response in all these systems for all the three different interaction strength regimes (Figure 2.7B). However, it appears that the response-type becomes signal strength dependent over the evolutionary period as low input signal responses develop sustained behavior (Figure 2.7B, rightmost panels). For convenience I show only the result of System AI. This result appears completely different from our previously published result (where all types of signaling molecules have an identical and fixed concentration) where the output

response is always sustained. From this observation, I conclude that the difference in the concentration of the signaling molecules are responsible for producing the transient cellular response. Note that the transient response in the STNs is not due to degradation of molecules (which is impossible in our STN) or explicit/manual introduction of negative feedbacks(manuscript submitted).

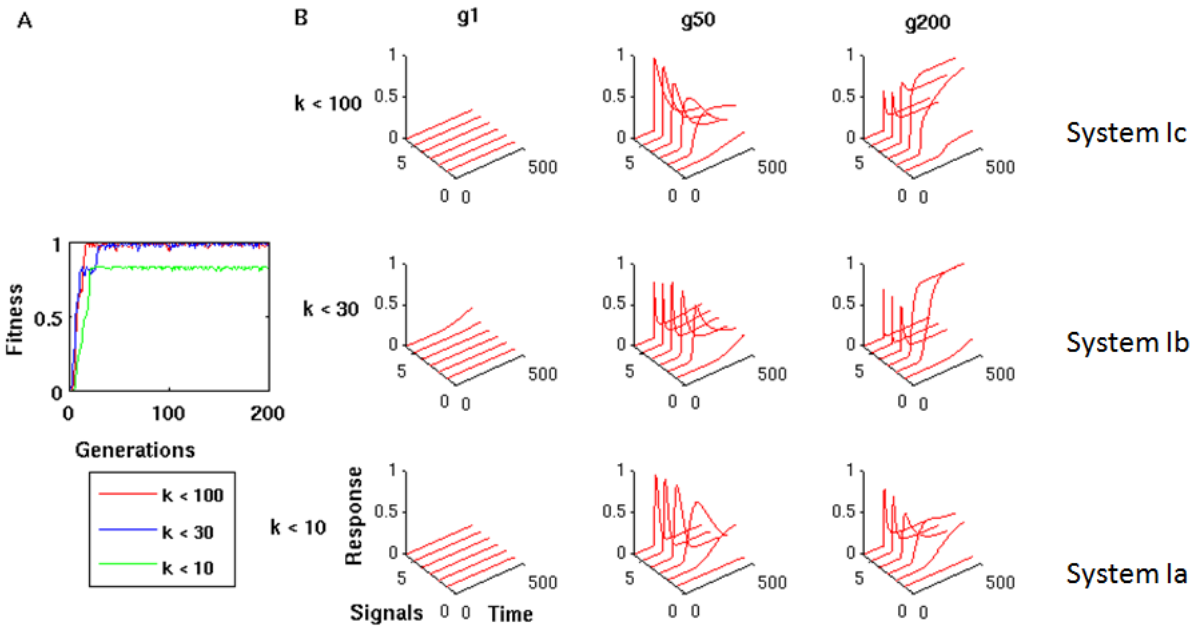

Figure 2. 7 Evolution of STNs by mutating kinetic parameters. The fitness (F_{norm}) during the evolution of STNs for three different regimes of maximal interaction strengths: weak (k <10), moderate (k <30), and strong (k <100) interactions (Number of generations: 200, Number of STNs: 200, Threshold level: $1/10^{th}$of the initial concentration of protein). For all the systems, six different strength input signal were used which are 10^{-7}, 10^{-5}, 10^{-3}, 0.1, 10, and 100. The concentration for the receptor, mediator, and effector molecules are 10, 5, and 1, respectively. (A) Evolution of STNs and (B) kinetics of the evolved STNs for three different kinetic parameter regimes in generation 1, 50, and 200. g1, g50, and g200 stand for generation 1, 50, and 200, respectively. The fitness shown here represents the mean fitness values of the fitness (F_{norm}) in the respective generations.

2.4 Effect of variation in the concentration of the signaling molecules on the kinetics of the STN response

To reveal more details about the effect of concentration variations in the receptor, intermediate, and effector molecules I have analyzed the kinetics of the systems II (variation in the concentration of receptor molecule), III (variation in the concentration of intermediate molecule), and IV (variation in the concentration of effector molecule). I found that in these three systems too, fluctuations in the concentration of SMs also lead to transient response for all the three regimes of kinetic parameters (Figure 2.8B, 2.9B, and 2.10B). The difference between the kinetics of the evolved networks of system I with the kinetics of the evolved networks of the other three systems is that in system I, most of the evolved networks show transient response while in other three systems (II, III, and IV) there are considerable number of the networks with sustained response. During the evolution (in generations 50 to 150) such evolution pattern of the kinetics in the evolved networks continues while by the end of evolutionary time i.e., in 200^{th}generations most of the evolved networks are with sustained responses which is prominent in particular for systems with strong interactions (Figure 2.8B, Figure 2.9B, and Figure 2.10B).

In our previous section (section 2.2), I have already shown that in the presence of equal and fixed concentration, the networks do not show any kind of transient behavior when the interaction strength varies during the evolution. In contrast in systems I (where the concentration is fixed but not equal and the kinetic parameters change during evolution), after 10 generations, I observed a transient activation pattern (Figure 2.7B). From these observations I conclude that the strength of input signals and the protein-protein interaction strengths do not play relevant role, neither together nor independently, in controlling the transient nature of the final response. Yet stronger input signals and stronger interaction strengths robust and sustained activation pattern. The factor left for inducing the transient response is the variation in the concentration of the signaling molecules or in other words the difference in the relative concentration of receptor, intermediate, and effector molecules(manuscript submitted).

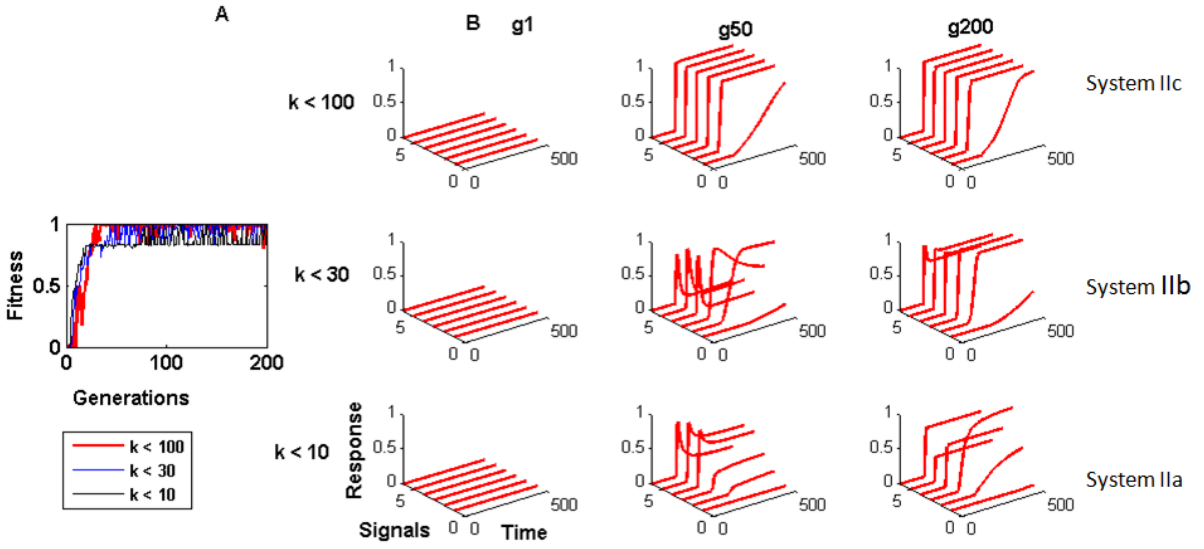

Figure 2. 8 Evolution of STNs by mutating kinetic parameters and the concentration of the receptor molecule A_1.The fitness (F_{norm}) during the evolution of STNs for three different regimes of maximal interaction strengths: weak (k <10), moderate (k <30), and strong (k >30) interactions (Number of generations: 200, Number of STNs: 200, Threshold level: $1/10^{th}$of the initial concentration of protein). For all the systems, 6 different strength input signal were used which are 10^{-7}, 10^{-5}, 10^{-3}, 0.1, 10, and 100.The fitness shown here represents the mean fitness values of the fitness (F_{norm}) in the respective generations.

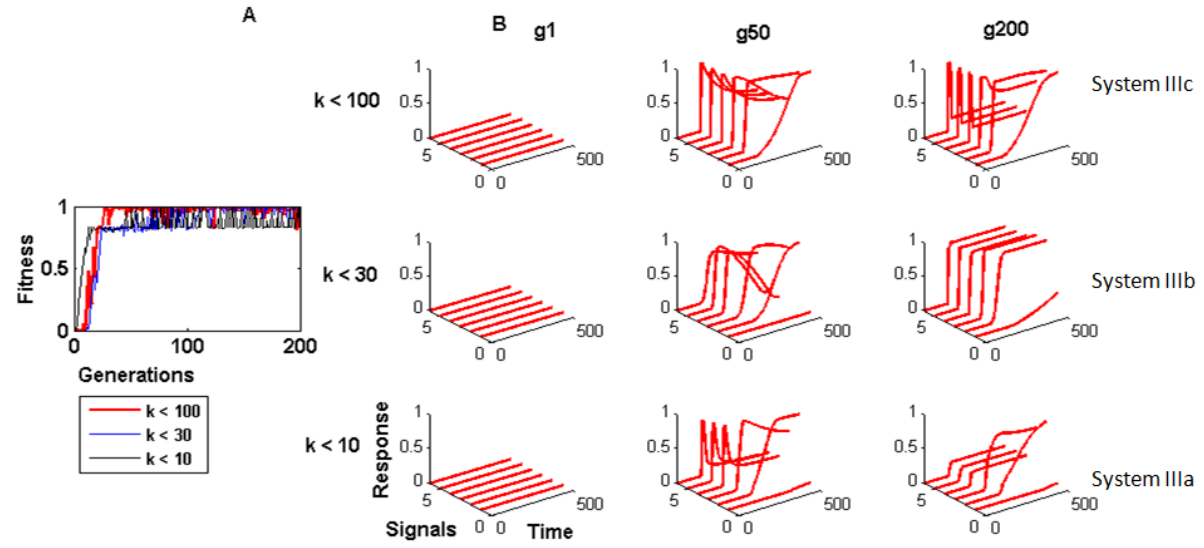

Figure 2. 9 Evolution of STNs by mutating kinetic parameters and the concentration of the intermediate signaling molecule A2 (A) Evolution of STNs and (B) kinetics of the evolved STNs for three different kinetic parameter regimes in generation 1, 50, and 200.The fitness shown here represents the mean fitness values of the fitness (F_{norm}) in the respective generations.

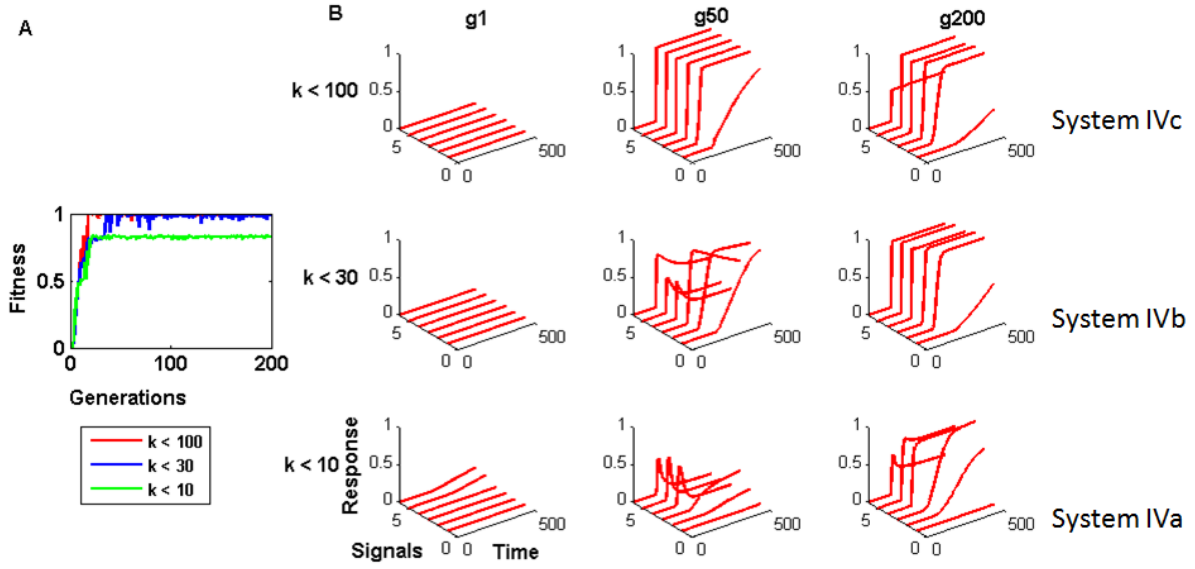

Figure 2. 10 Evolution of STNs by mutating kinetic parameters and the concentration of the effector molecule A3 (A) Evolution of STNs and (B) kinetics of the evolved STNs for three different kinetic parameter regimes in generation 1, 50, and 200.The fitness shown here represents the mean fitness values of the fitness (F_{norm}) in the respective generations.

2.5 Experimental verification of the effect of variation in the concentration on the final cellular response

To confirm theoretical findings (difference in the concentration of SMs as one of the possible factor for controling the transient response), Dr. Tina M. Schnöder used the pro-B cell line Ba/F3 stably transfected with the erythropoietin receptor(EpoR) and wildtype JAK2 tyrosine kinase. This cell line is completely dependent on the growth hormoneerythropoietin (EPO). Phosphorylation of several tyrosine residues of the cytoplasmic tail of the EpoRafter EPO stimulation leads to subsequent activation of intracellular signaling pathways including STAT5,MAPK, and PI3K/AKT [24]. Ba/F3 cells were serum-starved for 4 hours and then re-stimulated withdifferent EPO concentrations (0, 0.1, 0.5, 1, 2 U/ml) for various time points (5 min, 10, min, 30 min). As read-out for EPO signaling I analyzed the phosphorylation status of MEK and ERK1/2 (Figure 2.11). InFigure 2.11, I have shown that the pERK2 (upper band pointed with blue arrow) is transient while pERK1expression is sustained(Figure2.11A). If I compare the total amount of the two ERK isoforms I observe that ERK2 isfar less abundant than ERK1. Hence, in accordance with my results the different total amount correlateswith a

different kinetics, i.e. transient vs. sustained. Of note, the upstream activator for both ERK isoforms,the kinase MEK, shows a sustained pattern of activation (Figure2.11B) and could be considered as the signalthat is continuously present as in our simulations. For a full formal comparison with my results it would berequired, to fully quantify all other molecules interacting with ERK as well as the demonstration that theyhave similar kinetic parameters towards both ERKs. These experimental observations are suggestive to theidea that lower total concentration of a signaling molecule favors transient response. This could be furthertested using other cells with dissimilar amounts of two closely related isoforms that are dependent on thesame upstream activator(s) (Figure 2.11). The quantification of the western blot results have been shown in Figure 2.12.

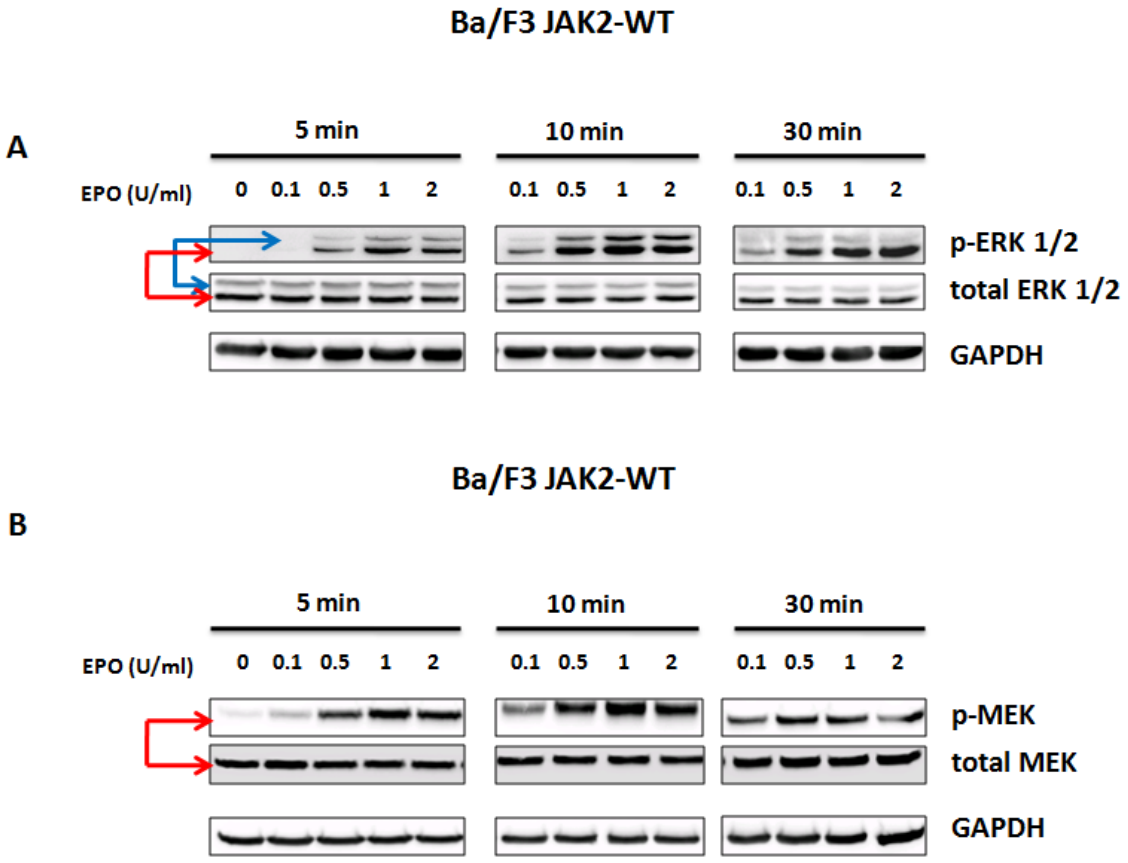

Figure 2. 11 Western blot analysis in Ba/F3 JAK2-WT cell line. Ba/F3 cells were cultured in RPMI1640 medium (PAA) supplemented with 10% fetal bovine serum (PAA) and with 1 U/ml of human recombinant Erythropoietin (EPO) (Janssen-Cilag) in a humid atmosphere of 5% CO2 at 37 °C. (A) pERK expression and (A) pMEK expression.

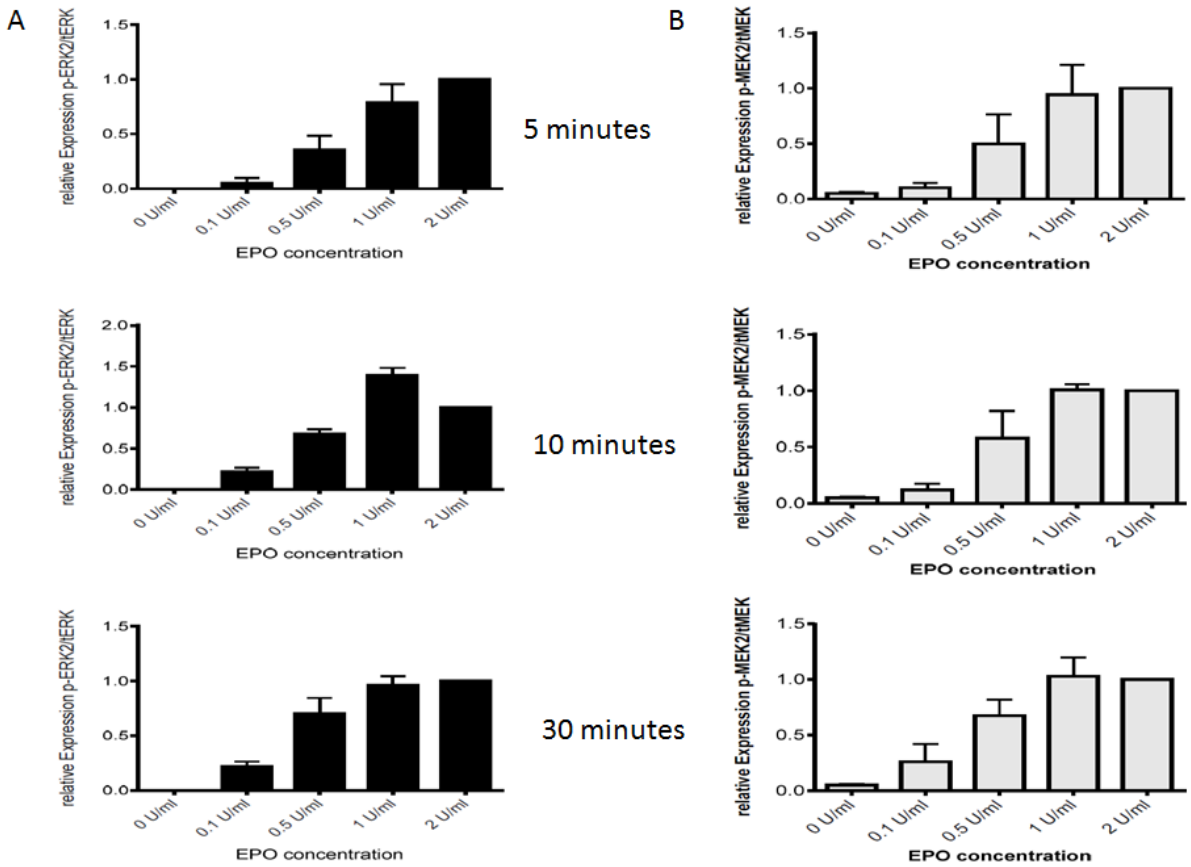

Figure 2. 12 Quantification of western blot analysis in Ba/F3 JAK2-WT cell line. This figure shows the quantification of the western blot analysis for the relation expression of (A) pErk1/2 and (B) pMek1/2.

2.6 Dose-response relationship of the evolved SNs

I have also investigated the relationship between the input signals and the output response in the evolved STNs. For this purpose, I considered ten input signals of different ranges (from weak to strong) beyond the range of our six signals used for selection. STNs were evolved as described in the previous sections and the different generations were tested for their response to the extended range of signals. The activation patterns evolving when strong interactions are permitted appear independent of the input signal strength, a behavior also known for the MAPK cascade in certain systems. In the beginning of the evolutionary time period (i.e., generations), irrespective of the input signals, all the networks have cellular response below the threshold level. After a few generations, the evolved STNs at very weak input signals, have their responses below the threshold level while for all other input signals, all the networks show strong activation even at comparatively weak input signal strengths (Figure 2.13). This means, the networks require a signal to become active confirming that the evolution did not generate self-activating networks, which would not be excluded by our choice of the fitness function. Thus, the strength of the input signal does not affect the peak response the SN. Therefore, it appears that the generic behavior of a SN is switch-like when facing the task to 'somehow' detect a signal. For all the systems (Figure 2.13), ten different strength input signals were used which are 10^{-9}, 10^{-7}, 10^{-5}, 10^{-3}, 10^{-2}, 0.1, 1, 10, 50, and 100(manuscript submitted).

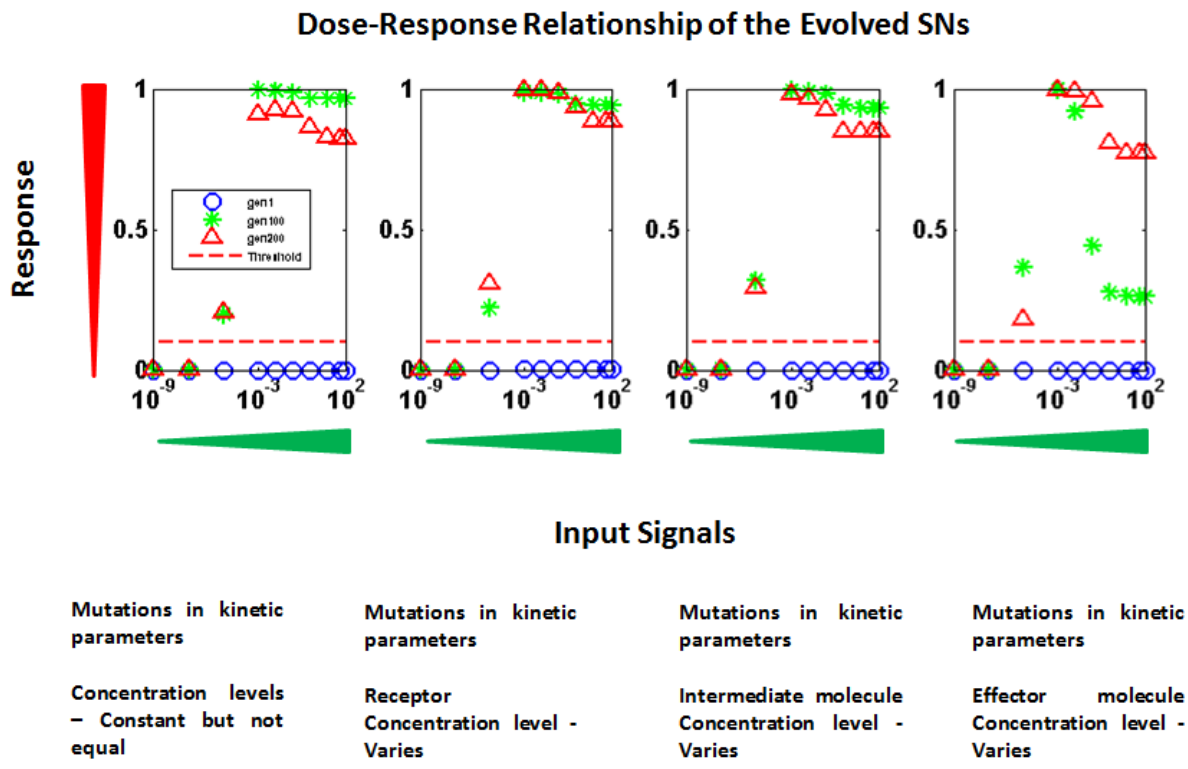

Figure 2. 13 Dose-response relationship of the evolved STNs for moderate interaction strength for all the systems The graph shows the maximum response of the output node of the best network versus the input signal in the respective generations 1, 100, and 200. For all the systems, 10 different strength input signal were used which are 10^{-9}, 10^{-7}, 10^{-5}, 10^{-3}, 10^{-2}, 0.1, 1, 10, 50, and 100.

2.7 Effect of removal of input signals on the kinetics of the evolved SNs

To further investigate the behavior of the output node, I simulated pulse activation of the networks: After initial equilibration of the network the signal is present for a fixed amount of time before being removed again. Before the pulse is given, all the networks remain in their basal inactive state. Evolved networks typically respond with a switch-like response to the signal pulse (Figure 2.14). Some of the networks will show an even enhanced response after the signal has been removed (Figure 2.14), while the majority revert back to the initial state. Since, our fitness function does not generate selection pressure to either of the network responses after removing the signal, both response types are valid. It is interesting to observe the occurrence of a pulse-detector, which requires a memory of previous signals, e.g. by generating irreversibility in the system. With a fitness-function sensitive to the phase following the removal of the signal, a trigger or an irreversible switch could be easily selected from the networks generated in our simulations (data published [23]).

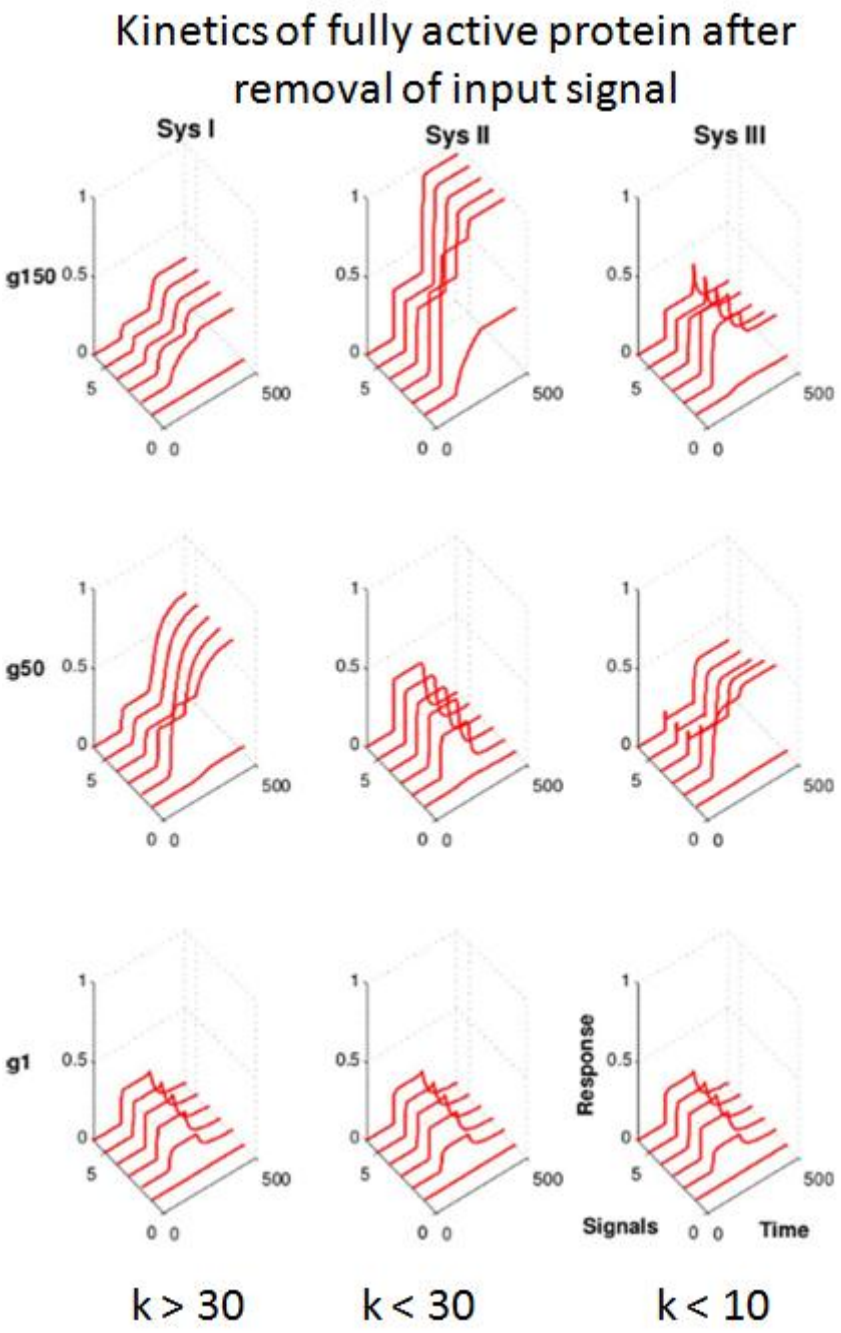

Figure 2. 14 Effect of the removal of the input signal on the kinetics of evolved networks. Shown are the networks with the highest fitness score from one representative simulation run. g1, g50, and g150 denote the generation 1, 50, and 150, respectively. The six solid lines show the kinetics of activation of the output node in response to six different input signal strengths (strength increases with signal index). Sys I, weak interactions (k<10). Sys II, moderate interactions (k<30). Sys III, strong interactions (k>30).

2.8 The role of partially active nodes

After studying the kinetics of the pre-defined output node (a fully active node) I also studied the kinetics of the partially active proteins. I observed that single phosphorylated proteins show predominantly a transient response and some of the networks shows partially adapted response (Figure 2.15). There is a clear trend that weaker interaction permit stronger transient activation of the monophosphorylated forms (Sys I, II, & III in Figure 2.15) (data published [23]).

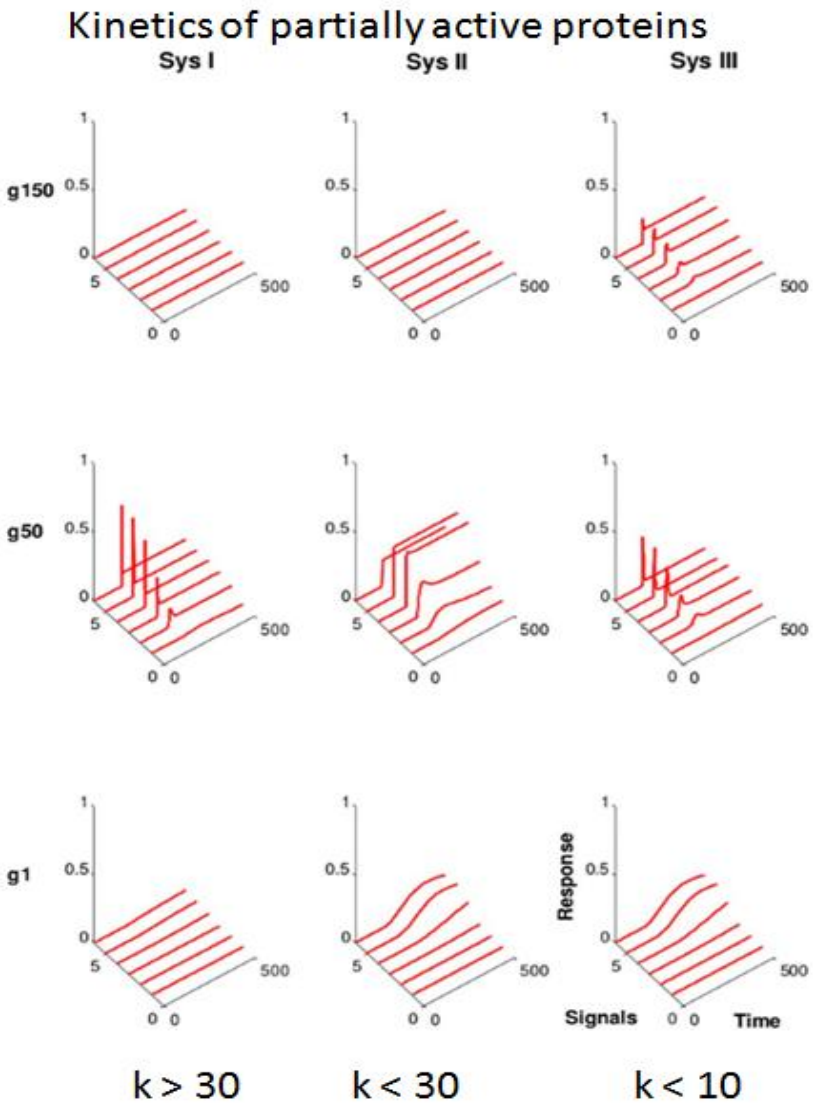

Figure 2. 15 Role of the partially active nodes. Shown is the kinetics of the node (A2p) from the networks with the highest fitness score from one of the simulation runs. g1, g50, and g150 denote the generation 1, 50, and 150, respectively. The six solid lines show the kinetics of activation of the output node in response to six different input signal strengths (strength increases with signal index). Sys I, weak interactions (k<10). Sys II, moderate interactions (k<30). Sys III, strong interactions (k>30).

2.9 Evolution of SNs by adding new proteins

In thenext step, randomly generated SNs were evolved by adding new proteins instead of altering only kinetic parameters. The new proteins are randomly interacting with potentially all proteins in all states (but not complexes) with also randomly generated kinetic parameters. Hence, all kinetic parameters are fixed as soon as the proteins are added. All other parts of the evolutionary algorithm remain the same. The addition of new nodes displays virtually the same effects on the evolution of the networks as well as the dynamics of the response which I observed due to the mutation of kinetic parameters. After a few generations, all evolving networks show similar and strong activation patterns provided the new interactions arising with the newly added proteins are of sufficient strength (data not shown). Also the distribution of the kinetic parameters of the new nodes shows no trends towards a particular pattern (data published [23]).

2.10 Cross-talk of the signal transduction pathways

For investigating the roles of the possible cross-talk (between different signaling pathways) on the cellular response, I have created two signal transduction pathways and randomly connected the one molecule of a pathway with another molecule of the second pathway. For example in cross-talk (Figure 2.16 e and f), one pathwaypositively/negativelyinteracts with the other pathway. After connecting two pathways, I have applied the evolutionary approach in the similar way as applied for investigating the roles of the kinetic parameters and the concentration of the SMs (mentioned in beginning of result section). Then I have analyzed the kinetics of the output response (response 1 for pathway 1 and response 2 for pathway 2).

In the past, many research groups have focused on the signal transduction pathways and investigated different factors which may play critical roles in controling the cellular response nature and finally the cell-fate (or cell-fate decision)[25-28]. The factors which have been investigated so far are the rate of reactions[13,23], network topology[13], concentration of the SM[29,30], feed forward loops (FFLs), feedback loops (FBLs) [25,26], or the cross-talk of the signal transduction pathways[17,18,31-36].

In biological systems, mainly four different types of cross-talks ((i) concomitant signaling, (ii) collaborative signaling, (iii) direct signaling, and (iv) amplification of signaling), have been reported[17]. Unlike to these previous works, I have started the investigation of a minimal cascade to the complex signaling regulation by adding all the possible interactions in one model.

Some of the FBLs[26], FFLs[37], and cross-talks[17,18,31,38-40] have been in investigated in biological signaling. In addition to these previously studied possible regulations, I have included more possible FFLs (both positive and negative), FBLs (both positive and negative), the combination of FFLs and FBLs, and increased more cross-talk possibilities (both the cross-interactions between the cascades i.e., inhibition and activation) between the linear cascades in one model and investigated their impact in controling the cellular response nature. From our results, I conclude that FBL and cross-talk plays critical role in determining transient cellular response. This model will help to understand the cellular response nature, to further reveal the new interactions based on the desired output response, and to perturb the output response by targeting the specific SM.

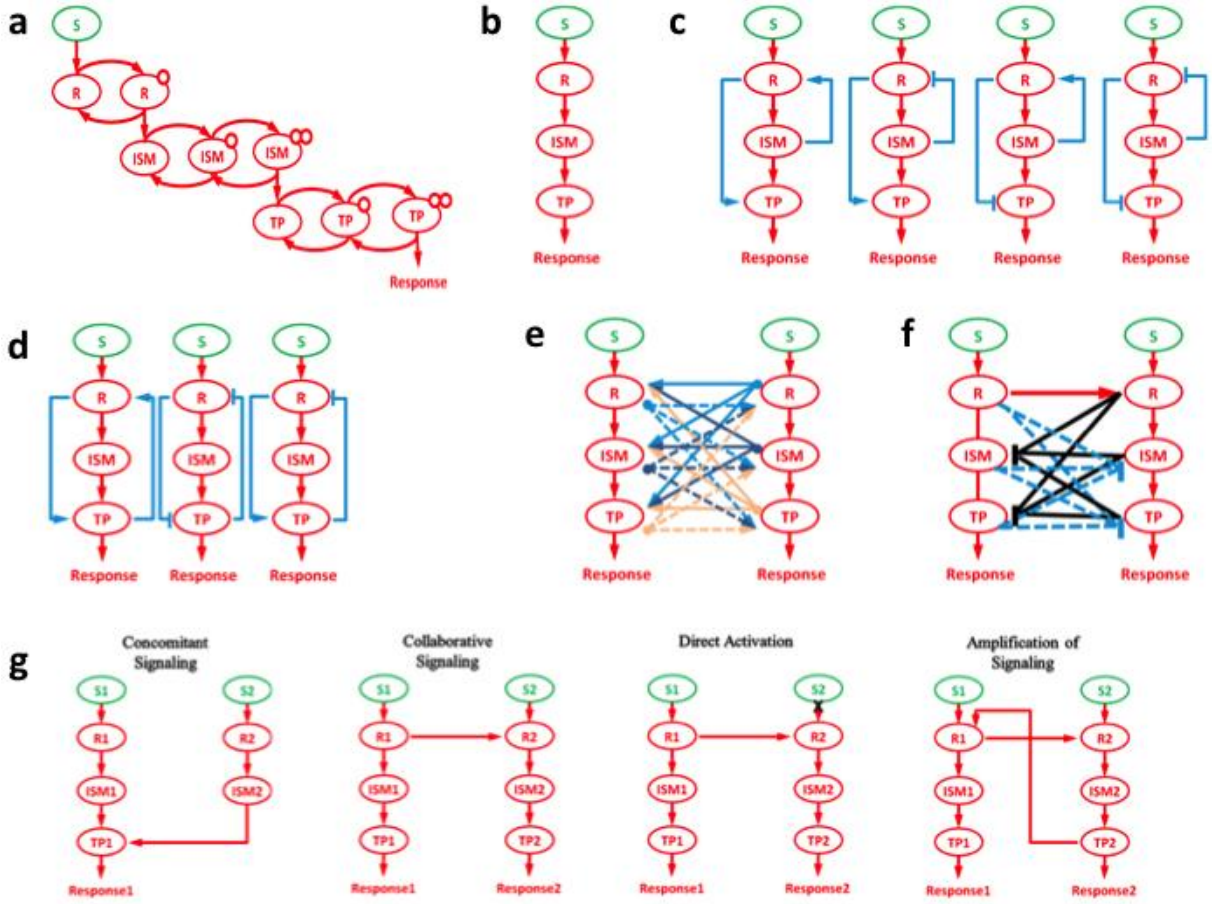

Figure 2. 16 Signaling cascade and its regulations. S, R, ISM, and TP stand for input signal, receptor, intracellular signaling molecule, and target protein, respectively. (a) A typical linear signaling cascade where R after detecting input signal S becomes active (goes to post-translational modification (e.g., phosphorylated)), active R activates ISM (single or double phosphorylation) and finally active ISM activates TP (single or double phosphorylation), (b) its simplified form, and (c) and (d) represents possible feed forward and feedback regulation (both positive (arrow) and negative regulation (blocked line)). (e) and (f) represent the cross-talks (arrows – activation and lines with blocked end -- inhibition) between signal transduction pathways (cascades). (g) cross-talks known in biological signal transduction [17].

As mentioned in the previous section, some of the FBLs, FFLs, and cross-talks have been in investigated in biological signaling. In addition to these previously studied possible regulations, I have included more possible FFLs (both positive and negative), FBLs (both positive and negative), the combination of FFLs and FBLs, and increased more cross-talk possibilities (both the cross-interactions between the cascades i.e., inhibition and activation) between the linear cascades in one model (Figure 2.16 a, b, c, d, e, f, and g) and investigated their impact in controling the cellular response nature. The major difference between the previous works and our work is the investigation of the combinations of different kinds of FFLs and FBLs and more cross-interactions between the signaling cascades in the presence and absence of FFLs and FBLs than the four positive cross-talks (Figure 2.16 g) reported by Ivaska J and Heino J [17,18,31,32,35,41-46]. In this model, the complex signaling networks have been simplified and represented as receptor level (R), intracellular signaling level (ISM), and target level (TP). So that the effect of different kinds of interactions at different levels on the final cellular response nature can be studied.

A linear cascade always produces sustained cellular response

Here, I have investigated the kinetics of the signaling molecules for linear cascade (a cascade without feed forward loop, feedback loop, and cross-talk between a pair of linear cascades) and linear cascades with feed forward loop and feedback loop (Figure 2.16 a, b, c, and d).

For this purpose, I have generated linear cascades with different sets of kinetic parameters (k_{par}). In case of signaling networks, the unit of k_{par} can be second^{-1} or minute^{-1}[47]. It is known that in general, the signal transduction process is faster than the other regulatory processes such as transcription networks and metabolic networks[48]. Throughout our work, I have written time instead of second or minute. Initially, k_{par} were randomly generated between 0.001 to 0.1. So, all the cascades have response kinetics close to zero (Figure 2.17a). Then, I have applied an evolutionary algorithm (EA)[23,49] to evolve the cascades. During the evolutionary period, I allowed the change in k_{par} and the concentration level of SMs. In this period, the signaling cascade adapts the improved kinetic parameters to produce better response.

After analyzing the kinetics of the evolved networks, I observe that in a linear signaling cascade (without any FFL/FBL), the change in the kinetic parameters or the concentration

does not produce any transient response (Figure 2.17 b, c, and d). Increase in the concentration (SMs) or the kinetic parameter values leads to improved sustained response (Figure 2b, c, and d).

Addition of a positive FFL in a signaling cascade (Figure 2.16c) does not change the cascade response and it remains sustained (Figure 2.17e, left) while the addition of a negative FFL disturbs the output response. The addition of a negative FFL produces mixed response either as transient, or sustained, or complete blocking of the response (Figure 2.17e, right) which means the activation pattern is not robust. Addition of FBL (positive or negative) leads to transient response (Figure 2.17f). Presence of one positive FFL and a positive FBL leads to sustained response (Figure 2.17g, left), presence of one negative FFL and a negative FBL and one negative FFL and a positive FBL leads to transient response (Figure 2.17 g and h), and presence of one positive FFL and a negative FBL does not change the sustained response to transient nature (Figure 2.17i). These FFL (positive or negative) and FBL (positive or negative) are from R to TP or TP to R (Figure 1d). When I apply the FBL (positive or negative) and/or FFL (positive or negative) from R to ISM or ISM to R in a cascade, I always observe sustained output response (Figure 2.17j).

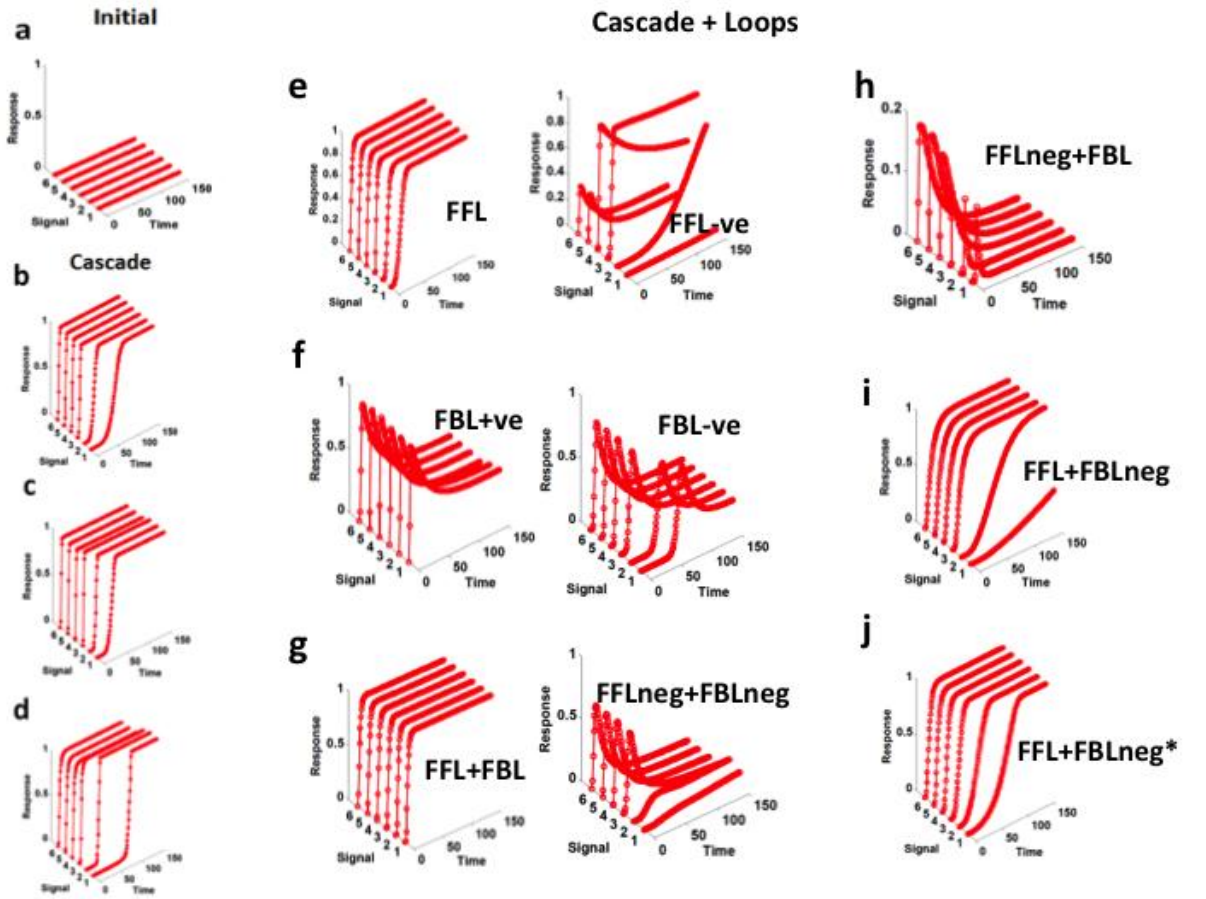

Figure 2. 17 Response kinetics (normalized value) of the signaling cascade. (a) Initially, kinetics of all the signaling cascade with or without additional regulation (e.g., FFL, FBL, or cross-talks) stays close to zero ($\boldsymbol{k_{par}}$ are generated randomly between 0.001 and 0.1). (b) kinetics of the fully evolved signaling cascade (the concentration of R, ISM, and TP are fixed and equal i.e., 10µl, during the evolutionary period the signaling cascades were allowed to adapt new $\boldsymbol{k_{par}}$ (between 0.1 and 100) values to improve the kinetic response). (c) kinetics of the fully evolved signaling cascade (the concentration of R, ISM, and TP are fixed and unequal i.e., 10 µl, 5 µl, and 1 µl, respectively, during the evolutionary period the signaling cascades were allowed to adapt new $\boldsymbol{k_{par}}$ (between 0.1 and 100) values to improve the kinetic response). (d) kinetics of the fully evolved signaling cascade (initially the concentration of R, ISM, and TP are fixed and equal i.e., 10 µl, during the evolutionary period the signaling cascades were allowed to adapt new $\boldsymbol{k_{par}}$ (between 0.1 and 100) values and change in the concentration of R, ISM, and TP to improve the kinetic response). (e) kinetics of the fully evolved signaling cascade in the presence of FFL (the concentration of R, ISM, and TP are fixed and unequal i.e., 10 µl, 5 µl, and 1 µl, respectively, during the evolutionary period the signaling cascades were allowed to adapt new $\boldsymbol{k_{par}}$ (between 0.1 and 100) values to improve the kinetic response). (f) kinetics of the fully evolved signaling cascade in the presence of FBL, (g) FFL and FBL (both positive and negative), (h) negative FFL and FBL, (i) FFL and negative FBL, and (j) FFL and negative FBL from ISM to R (the concentration of R, ISM, and TP are fixed and unequal i.e., 10 µl, 5 µl, and 1 µl, respectively, during the evolutionary period the signaling cascades were allowed to adapt new $\boldsymbol{k_{par}}$ (between 0.1 and 100) values to improve the kinetic response).

Concomitant inhibitory between cascades dominantly produce transient response

After analyzing the kinetics of signaling cascade response, I investigated the change in the kinetics of the TP of the signaling cascade in the presence of different kinds of cross-talks known from biological system (reference). I have investigated their inhibitory forms (in biological cross-talks the links between the cascades are activation) also for all the four cross-talks. I found that concomitant signaling (activation link between two cascade) leads to sustained response (Figure 2.18a) and its inhibitory form produces transient response (Figure 3b). While all the three other kinds of cross-talks (collaborative, direct, and signal amplification) between the cascades help in producing stable sustained response (Figure 2.18 c, d, e, f, g, and h) irrespective the nature (activation or inhibition) of the links between the cascade. In case of direct signaling, inhibitory interaction between the two cascades leads to only one output response in cascade 1 and complete blockage of the output response of cascade 2 (Figure 2.18f) because here input signal (S_2) is blocked.

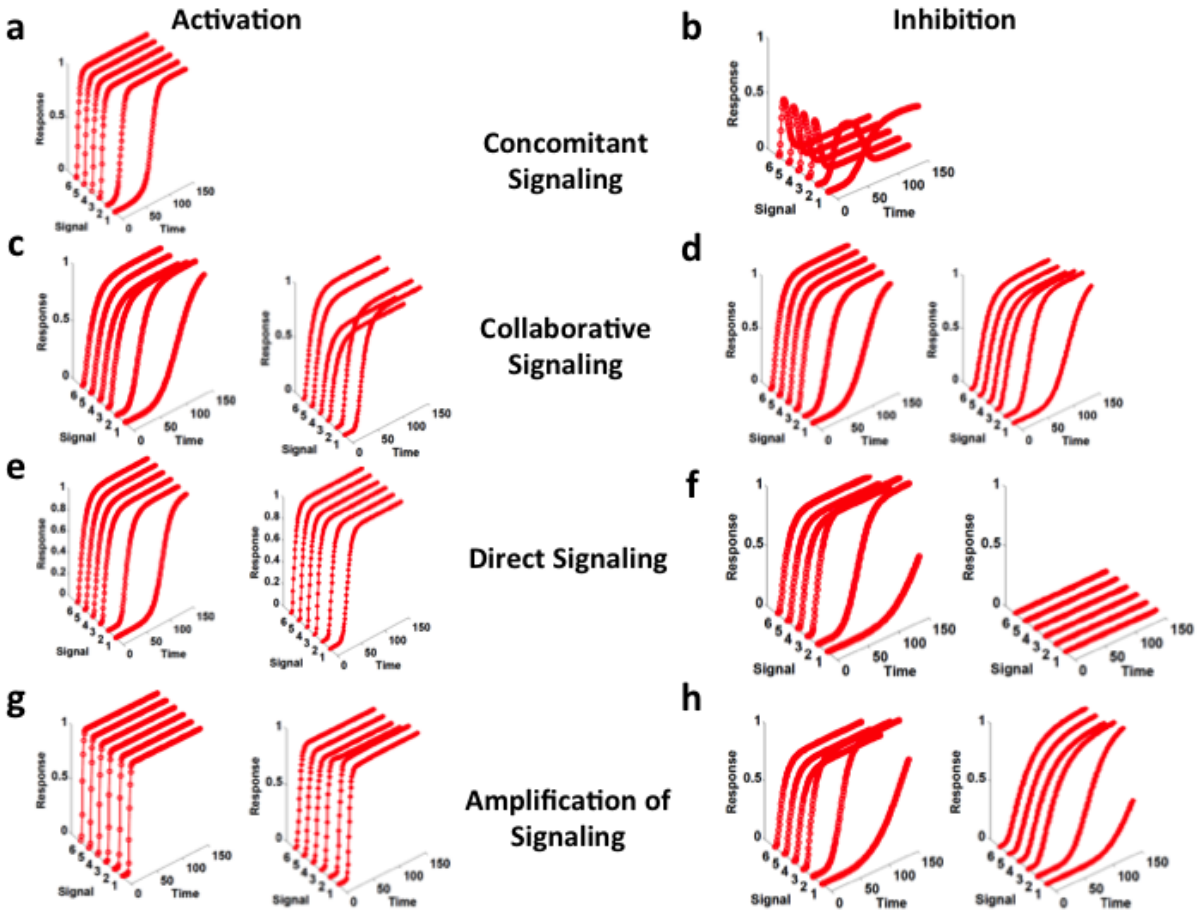

Figure 2. 18 Kinetics of output response in case of cross-talk between the signaling cascades. (a) activation concomitant signaling, (b) inhibitory concomitant signaling, (c) activation type collaborative signaling, (d) inhibition type collaborative signaling, (e) direct signaling – activation, (f) direct signaling – inhibition, (g) amplification of signaling – activation, and (h) amplification of signaling – inhibition. In figure c, d, e, f, g, and h, left side figure represents the kinetics of the output response of cascade 1 and right side figure represents the kinetics of out response of cascade 2.

Increase in the number of inhibitory links leads to transient response or complete blockage of the output response

Finally, I have investigated the effect of all the possible interactions (FFL, FBL, and cross-talks) in a single model. Here, I have two linear cascades in parallel without any cross-interaction. I have generated 200 sets of parallel cascades and evolved them in parallel until 100 generations by allowing the rate of reactions (k_{par}) to change during evolutionary period to adapt new k_{par} in order to produce improved kinetic response. After 100 generations, all the new interactions were added one-by-one in a linear signaling cascade (Figure 2.16a) in each generation. In this work, first I have started addition of negative interactions between two cascades, then FFL and FBL, and finally the positive interactions between cascades.

I observe that all the minimal cascades produce sustained output response for all the six different (strength) input signals (Figure 2.19 a and b). In contrast, addition of new inhibitory interactions between the two cascades, FFL, and FBL leads to transient response which can be seen between generation 100 and 165 in Figure 2.19 a and b. The response nature has been shown in Figure 2.19c (for a linear cascade – where both the cascades produce sustained output response before generation 100 (left – pathway 1 and right – pathway 2)). Since, in the beginning I add the interactions through which pathway 2 inhibits pathway 1 so the output response of pathway 1 is transient and pathway 2 remains sustained (Figure 2.19d). When I add the interactions (inhibitory) between both the pathways then both the pathways produce transient response or completely block the output response of both the pathways (Figure 2.19e). Addition of positive interactions between the cascades lead to the sustained output response which can be seen in Figure 2.19 a and b after generation 165 and the kinetics of the output appears similar to Figure 2.19c. As far as the fitness of the cascades is concerned, as long as the cascades are free from additional interactions, the fitness remain stable and stays at maximum (Figure 2.19f (left)) because the kinetics of all the cascades for all the input signals easily crosses the threshold level and remain sustained. While addition of new inhibitory interactions between the cascades and the FFL and FBL shows fluctuation in the fitness because the output response becomes either transient or does not crosses the threshold. I further investigated the change in the k_{par}. In linear cascade which has comparatively less number of reactions so the mean of the k_{par} is

comparatively lower than the cascade with new interactions and the addition of new interactions in each generation leads to the gradual increase in the k_{par} (Figure 2.19f (right)). So, I conclude that the irrespective the response nature (sustained or transient) the k_{par} increases but it does not affect the cellular response nature.

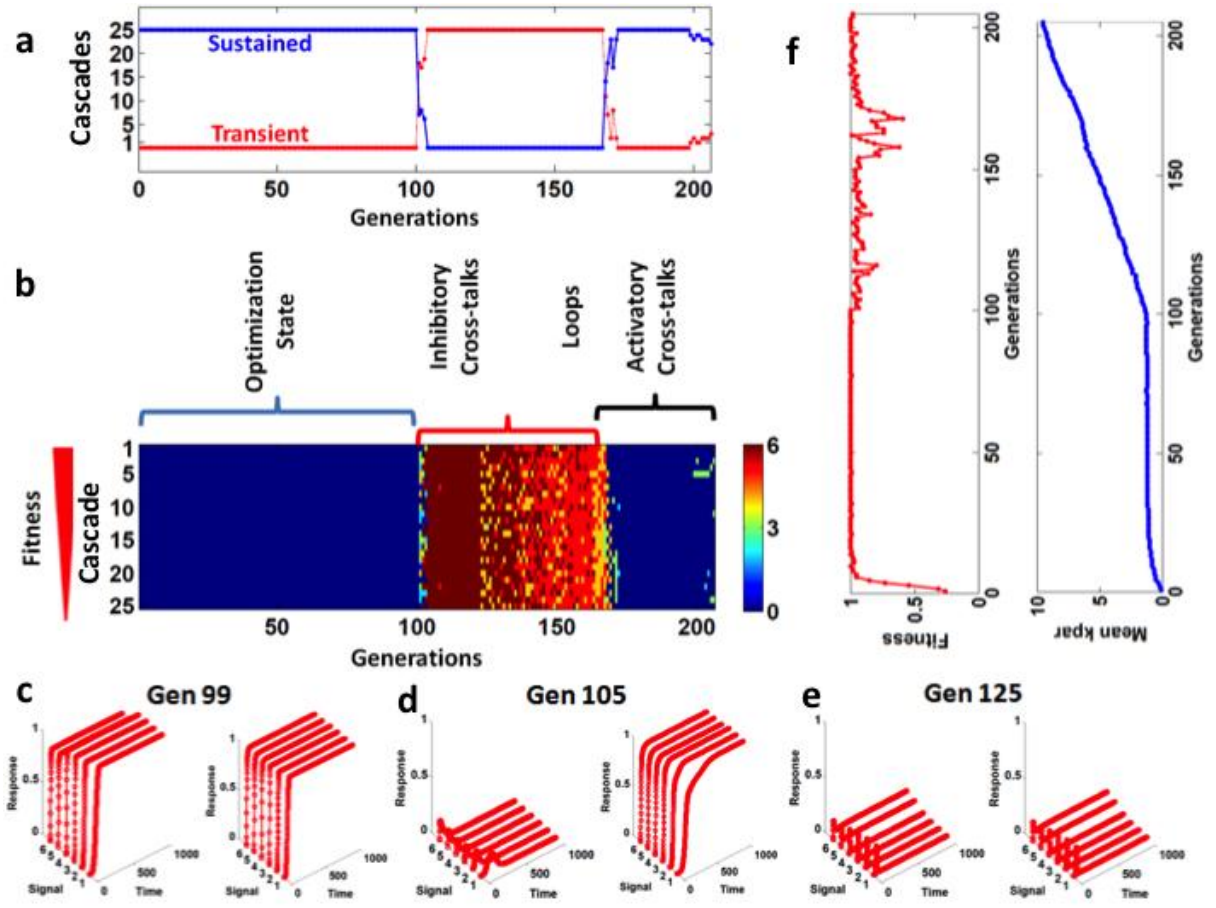

Figure 2. 19 Change in response kinetics of the signaling cascade from simple cascade (without FFL, FBL, and cross-talk) to complex cascade (with FFL, FBL, and cross-talks). (a) Total number signaling cascades with transient and sustained response among the best 25 signaling cascades. (b) As in our model for each cascade I have six input signals (of different strength) so I have six output response. Here, I show total number of response (transient and/or sustained) in each cascade. (c), (d), and (e) show the kinetics of the output response (cascade 1(left side figure) and cascade 2 (right side figure)) in generation 99, 105, and 125, respectively. (f) Mean fitness (left side) and the mean of $\boldsymbol{k_{par}}$ (right side) of cascades (best cascades).

Chapter 3 DISCUSSION

In my thesis work, I have investigated the roles of input signal and its strengths, kinetic parameters, addition of the new nodes, concentration of the SMs, and the cross-talk of the signaling pathways on the cellular response.

Inthe previously published work, four different possible input-output relations for signal transduction have been shown[50]. The first one is the classical case which is single input and single output, the second possible relation is signal concatenation (multiple inputs and single output), the third relation is signal pleiotropy (single input and multiple outputs), and last one is the complex signaling event which has multiple inputs and multiple outputs. Out of all these four possible signaling events my model is designed to represent the classical case which is frequent in biological signaling processes. Although not the scope of my present study,the model can also be modified to understand the remaining three types of signaling input-output relations in the future.

I haveinvestigated the evolution of STNs under the premise that the primary task of signal transduction is to detect a signal without pre-determining a desired kinetics. Any form of the protein can - depending on its interaction partner - play the role of a kinase or phosphatase. Typically, proteins do not fulfill both functions, however, due to their phosphorylation state may recruit proteins that perform this function but are not explicitly modeled in my approach. During the evolutionary process,the mutations were allowed either in the kinetic parameters or the topology of the network or in the concentration of the SMs. The STN population achieves maximum fitness only when protein-protein interactions are sufficiently strong.

When the SNs were evolved by allowing mutation in kinetic parameters only and the concentration of the SMs were fixed and equal, then the generic solution is a sustained activity of the output node as long as the signal is present. Weak interaction strength results in networks that respond differently and only to some of the signals. In a cellular system weak interactions could therefore probably not provide reliable cellular decisions. For the input sensor - response relationship, I conclude that neither the starting topology nor the set of kinetic parameter values is constraining the evolution of the networks (provided sufficiently strong interactions). Therefore, short circuits coupling the receptor directly to the output node by two reactions are possible but certainly not the only solutions. In

particular, the final generation has a high variability in the kinetic parameters suggesting that no dominant subnetwork (network motif) of interactions exists.

The activation pattern of the evolved networks in a stationary population are robust against strong interactions and in most cases sustained response, suggesting that this type of response is the generic cellular behavior when the presence of a signal is sufficient information for a cell[51].I observe that the response kinetics does not alter after about 30–50 generations but the kinetic parameters still change. This observation lead to theinterpretation in the following way: When the first networks with enhanced fitness appear they give rise to multiple clones that have largely similar kinetic parameters. This is similar to the bottleneck effect, i.e. many networks do not generate offsprings due to their low fitness and only similar networks pass on to the next generation. Following this phase the networks start to diversify again and a large range of the allowed kinetic parameter regime is explored. The diversification also indicates that there is a large number of solutions in the parameter space to 'solve' the fitness function. These solutions appear to be connected in a large set as the different kinetic parameters can be explored by the STNs without losing their fitness. Alternatively, one can view this situation as overfitting as the quite large number of parameters allows the networks to 'solve' the fitness function in many different ways.

In addition to above mentioned simulation condition (where the SNs were evolved by allowing mutation in kinetic parameters only and the concentration of the SMs were fixed and equal), I have evolved the SNs for four different conditions. In these simulations, mutations were allowed during the evolutionary process in the kinetic parameters and also in the concentration of the receptor, intermediate, and the effector molecules. The simulations, where the mutations were allowed only in kinetic parameters and the concentration of the receptor, intermediate, and effector molecules are fixed but unequal, has a transient output activity as generic solution. Irrespective of the constraints on the kinetic parameter almost all the networks are able to detect the signal and respond in a transient manner.

The evolved networks in a stationary population show stable activation pattern against strong interactions and the transient response in System I (Figure 2.7Bin section 2.3), suggesting that this type of response is the generic cellular behavior when the presence of a signal is sufficient information for a cell[51]. I observe that in System I the response kinetics

does not alter after about 30 to 50 generations and maintains transient nature but the kinetic parameters still change as shown in section 2.2 (Figure 2.4). While in case of change in the concentration (System II, III, and IV), a significant number of the evolved STNs show transient response within 100 generations. Afterwards the response kinetics fluctuate too much and a large number of STNs change their transient behavior to sustained behavior while the kinetic parameters and concentration still change.

I compared the evolved SNs for the system where the mutations were allowed in kinetic parameters only and the concentration of the SMs were fixed but equal with the evolved SNs for the system where the mutations were allowed in kinetic parameters only and the concentration of the SMs were fixed but unequal. For the former system, I have shown that the strength of output response is directly proportional to the kinetic parameter and the nature of response is always sustained. I have also shown that the final response of the evolved networks becomes stronger and stronger until they reach to the maximum and almost all the networks show sustained response(Figure 2.3). In the later system (where the mutations were allowed only in kinetic parameters and the concentration of SMs were fixed and unequal), in all the evolved networks I found that irrespective of the kinetic parameters the nature of final response is always transient (Figure 2.7B).

From this comparative analysis I conclude that the difference in the concentration of the receptor, intermediate, and effector molecules is responsible for this transient response (Figures2.7B, 2.8B, 2.9B, and 2.10B). In another way I can say that irrespective of the protein-protein kinetic parameters, difference between the concentration of signaling molecules (receptor, intermediate, and effector) acts as one of the possible factors for controling the transient response when the mere detection of the presence of a signal is relevant (System I, II, III, and IV).

Based on these results it appears that the difference in the concentration of the SMs as one of the possible factor leading output response to the transient behavior. Since the kinetic parameter and input signal strengths do not have any direct impact on the nature of the final response but when I evolved the networks allowing the variations in the protein concentration during evolution then I found that there are majority of the evolved networks which show transient response. So I can say from these observations that the fluctuation in protein concentration definitely play some kind of roles in controlling the transient output

response. In addition, I have also shown the experimental results to validate my theoretical findings of the effect of concentration difference on the final cellular response. Where the results clearly show the transient response due to the variation in the concentration of the signaling molecules (Figure 2.10).

The higher average fitness of networks with strong interactions is due to the ability to detect weak signals. This corresponds to situations such as bacterial chemotaxis and T-cell receptor signaling where cells are sensitive to detect very few ligands. In the simulated systems, I have three different simulating conditions. weak (k<10), moderate (k<30), and strong (k>30). Signaling networks are still functional with or moderate interactions. However, at weak interaction strengths the STNs will work but in case the input signal is also weak then STNs often fail to detect. When the interaction strength is moderate then the STNs function and can also respond to weak input signals. Another difference between the STNs working at moderate and strong interaction strength is that at strong interaction strength the STNs show a strong and quick output response in contrast to weak interactions where the kinetics is typically slow and weak.

The rapid increase in fitness for the STNs suggests that any weakly interacting network that is capable of evoking at least some response to a signal, quickly evolves into a strongly interacting STN provided the selective pressure is present. This results in a high flexibility of cells to gain new signal transduction pathways when required and the critical invention is the proper receptor rather than a correct connection to the appropriate cellular response. Thus, a cell may retain a number of weak interactions among signaling proteins that do not interfere with primary signaling pathways, which can be converted should such a demand arise during evolution. As a consequence, a high number of weak unspecific interactions among proteins enables the cell to flexibly and quickly adapt to changing environments. Based on our results, I hypothesize that this is not a property that must be developed by a cell during evolution, but is inherent to weakly interacting protein-protein interaction networks. The diversification of kinetic parameters following the evolution of successful STNs in the regime of strong interactions also indicates that a large number of weak interactions do not harm the performance of the evolved signal--response relation. Thus, the STNs can reliably respond to the signal while at the same time retain a plethora of connections which may be used to ‘solve’ evolutionary demands that may occur in addition.

This effect is in agreement with the notion that robustness combined with a high evolvability is a favorable and likely outcome of evolution [52-54].

I have also computed the signal-response relationship (peak response versus input signal) for all the four systems. For all these four different simulation conditions, the activation patterns in the evolved STNs when strong interactions are permitted appear independent of the input signal strength, a behavior also known for biological signaling systems. In the beginning of the evolutionary time period (i.e., generations), irrespective of the input signals, all the networks have cellular response below the threshold level. In the beginning of the evolutionary period, the STNs have too weak kinetic parameter to initiate the signaling reactions. After a few generations the STNs adapt new kinetic parameter to strong kinetic parameter. STNs at very weak input signals, have their responses below the threshold level (shown in Figure 2.13) while for all other input signals, all the networks show strong activation even at comparatively weak input signal strengths (Figure 2.13).

As I have discussed in section 2.1 to 2.5 the model which evolves STNs with the variations in kinetic parameter and concentration of the signaling molecules of the STNs[21,22]. The kinetic parameters of all the signaling molecules are in general hard to access experimentally by the experimentalists. In the simulations, I see that the exact value of the kinetic parameter plays a minor role in controling the transient nature of the final output response. Unfortunately, the number of possible parameter sets to generate a certain behavior is large such that the topology alone is not likely to predict the function of the network reliably. However, it is not the only kinetic parameters and input signal which vary but also protein concentration affect the behavior of a STN [21,22] and protein concentrations bear the advantage of being experimentally quite easily accessible variables. Furthermore, stimulation of receptors virtually never occurs in isolation, the triggered receptors activate the downstream signaling molecules in order to transduce the response to the nucleus. The activated signaling molecules can also activate the nearby SMs which are the part of another signal transduction pathway. Therefore, the interaction of signal transduction pathways can become relevant [50,55,56]. The questions of how does cross-talk affect the network's behavior and how does it affect the evolution of the STN is worth pursuing using our approach by embedding a STN into the wider context of co-evolving signaling networks.

To address the role of cross-talk of signal transduction pathways, I have investigated four different cross-talks and found that out of these four cross-talks (Figures 2.16, 2.17, 2.18,

and 2.19) only one of the cross-talk (a cross-talk where the fully active form of downstream SM A_{3pp} of pathway 1 dephosphorylates the partially active downstream SM B_{3p} of pathway 2 to the inactive form B_3shown in Figure 2.17 (called as cross-talk type 2)) has transient response for response 1 of pathway 1 while all other cross-talks produce sustained response. Based on the my current results related to cross-talk work, I can say that in addition to the difference in the concentration of the SM, few of the cross-talks may also play critical roles in controling the transient cellular response.

From previous experimental works [30,57] some interesting facts about the effect of variation in the concentration of SMs are known. Here, they have investigated the role of change in the concentration of an individual molecule and not investigated in comparison to the other molecules involved in signaling.

In the previous works[17], it has been shown that in a biological system there are different kinds of cross-talks (see section 1.4 cross-talk) which may occur during signal transduction process from receptor level to the nucleus (which means the interaction between the pathways can take place at different level (i.e., at the receptor or intermediate or effector level)). In my thesis work, I have set up a simplistic mathematical model which can theoretical investigate the effect of known cross-talks similar to the biological system and in the future, this model can be applied to understand the biological system. The advantage of our model is that it will not only help to understand the effect of the variation in the concentration of the receptor molecules but also help to understand the impact of the concentration of other signaling molecules (such as intermediate and effector molecules) involved in the signal transduction. These models can only be applied to those systems which are known to have such behavior, but often the exact behavior of the STNs is not known. Therefore, the creation of a fitness function that encodes the task that a cell solves under certain experimental conditions, may be more beneficial in determining possible and likely behavior of the underlying STNs.

In cross-talk of signal transduction pathway study, I have investigated the change in the output response nature (sustained or transient) of the signaling cascade in the presence and absence of the FFL, FBL, and cross-talks between two cascades. The cascade which I have used here, is similar to the MAPK cascade[58]. Based our data, I propose that transient signaling responses result from FBL and/or negative cross-interactions between signaling

cascades. If the concentration of the TP is lower than the concentration of the R and ISM, and either FBL or negative cross-talks are present then all the cascade produce consistently transient output response. Irrespective of the concentration of the signaling molecules, FFL and all the positive interactions (cross-talks) between the cascades lead to stable and sustained output response.

The evolved networks in a stationary population show stable activation pattern against the change in kinetic parametersfor both signaling cascades (until generation 100) and addition of the positive interactions between the signaling cascades (after generation 165 onwards) and the output response assustained response.This suggests that this type of response is the generic cellular behavior when the presence of a signal is sufficient information for a cell. While in the presence of inhibitory interactions between the signaling cascades and the cascade with FBL and the simultaneous presence of FBL and FFL, the kinetics of the output response is always transient (if the concentration of the TP is less than R, and ISM). The fitness of the cascades fluctuates significantly. This suggests the transient response as the generic solution. If the concentration of R, ISM, and TP is equal then the cascades with inhibitory cross-talks and the cascade with FBL or with combination of FFL and FBL also produces the transient response but not all the cascades (with the exceptions of few cascades having sustained response).

From previous works[17,25-27,59,60], some interesting facts about the effect of variation in the concentration of SMs, FBL, FFL, and cross-talk of signaling pathways are known. Here, they have investigated the role of change in the concentration of an individual molecule and not investigated in comparison to the other molecules involved in signaling. The FBL, FFL, or cross-talk of pathways have been investigated individually and not in combination of FFL and FBL or cross-talk.

Most of the complex and/or common diseases such as cancer, diabetes, obesity, and asthma are caused by defects in multiple genes and pathways. So, it is not surprising that the current one-target-one-compound approach in drug discovery and development has failed to deliver as many efficacious medicines as expected in the post-genomic era[38,61,62]. In order to understand such complex diseases and find therapeutic solution, it appears to be promising point to understand the signal transduction process from a simple linear cascade to a complex regulatory mechanism (a linear cascade with different loops and the cross-

interactions of the cascade) of signaling network.By applying this approach, I can selectively target the signaling molecules to get the desired output response and will help to target multiple signaling molecules.

The advantage of our model is that it will not only help to understand the effect of the variation in the concentration of the receptor molecules but also help to understand the impact of the concentration of other signaling molecules (such as intermediate and effector molecules) involved in the signal transduction and will give an insight of the different additional regulations such as FFL, FBL, and cross-talk. These models can only be applied to those systems which are known to have such behavior, but often the exact behavior of the STNs is not known. Therefore, the creation of a fitness function that encodes the task that a cell solves under certain experimental conditions, may be more beneficial in determining possible and likely behavior of the underlying signaling cascades.

3.1 Future Perspectives

As I have discussed that I have investigated the roles of the input signals, kinetic parameters, addition of new node (change in network topology), concentration of SMs, and four different cross-talks.Still there are many interesting points which can be done in the future by using the current approach and model.

Here, I discuss three points as the future perspectives:

- To implement cross-talk between the signal transduction pathways in biological system
- To investigate the roles of domains and motifs of the SMs and the scaffold formed by the SMs during signal transduction
- To investigate the signaling network motifs

3.1.1 Protein domains and motifs concept in signaling

In cell signaling, there is a huge number of multi-domain proteins known and these proteins play critical roles in signal transmission from receptor levels to the nucleus. These domains are of different nature like catalytic or regulatory (Figure 3.1). The signaling molecules also possess a set (pattern) of amino acid residues in particular pattern (for example: TCR chains contain ITAMs (immunoreceptor tyrosine-based activation motifs) as motifs with sequence of YXXL/I) known as motifs[63].

The domains typically contain >30 amino acid residues while motifs have < 10. The domains and motifs passes through different physical change (like phosphorylation) to activate/deactivate the signaling molecules for the respective cellular functions. It is also known that the different combinations of the domains (catalytic/interaction) and motifs together produce complex targeting and regulatory activity of the proteins [64-68].

To introduce the importance of the motifs during signal transduction I took the example from previous studies. It is known that ITAMs are the essential part of the TCR/CD3 complex which are phosphorylated by Lck to trigger the downstream signal processing. Recently, it has also been shown that distinct TCR signaling pathways drive proliferation and cytokine

production in T cells[63]. In addition to ITAM motifs, TCR/CD3 complex also possess additional motifs such as BRS (basic residue-rich stretch) and PRR (proline-rich region). BRS motifs plays important roles in membrane association of TCRζ cytoplasmic chain with the plasmamembrane and modulate T cell signaling. The PRR motif has no role in TCR triggering but is considered to be involved in the regulation of TCR expression levels in thymus[69].

For a more detailed understanding of the role of the protein domains in signal transduction, I take an example of the architecture of the Ill characterized Src family kinase (SFK) protein which contains the proteins Src, Lck, Fyn, Hck, Lyn, Blk, Fgr, Yes, and Yrk found in many cell types. Each SFK has four domains: the unique region, folloId by the SH3, SH2, and a kinase domain (SH1 domain). The unique region varies among the family members. For full activity, SFK members must need to be phosphorylated in the kinase domain at Tyrosine 416 (autophosphorylation site in Src) or its homologs in the other members of the Src family, respectively[70].

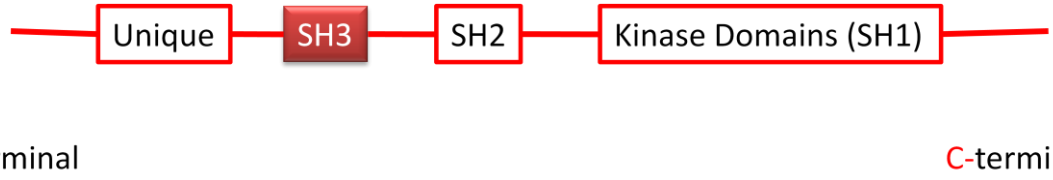

Figure 3. 1 Domain structure of Src family kinase proteins. Unique region varies among the SFK members, The unique region is folloId by SH3, SH2, and kinase domain.

- **Possible aim for Protein motifs and the domains:**

  In signal transduction, the signals are mediated by the networks of proteins. These signaling specialized proteins are made up of specific kind of substructures in the form of motifs and domains. As mentioned previously, these substructures play critical roles in modulating the signals. These domains and motifs also help the signaling molecules (present inside or around the cells) to combine each other to form a bigger complex structures which are known as scaffolds and adapters. The scaffolds and/or adapters assist in modulating the signals in much better way.

So, in this study I mainly focused on the structural properties of the signaling proteins, docking interactions, scaffolds, and adapters, second, the interactions betIen proteins, motifs, domains, docking interactions, scaffolds, and adapters (Figure 3.2). To investigate these properties of the signaling molecules, I have investigated the cellular response due to the (i) shuffling of the domains and motifs, (ii) mutation in the domain and motif sequence pattern, and (iii) the shuffling of the mutated domains and motifs or the shuffling of the mutated domain/motif with wild type domain/motif.

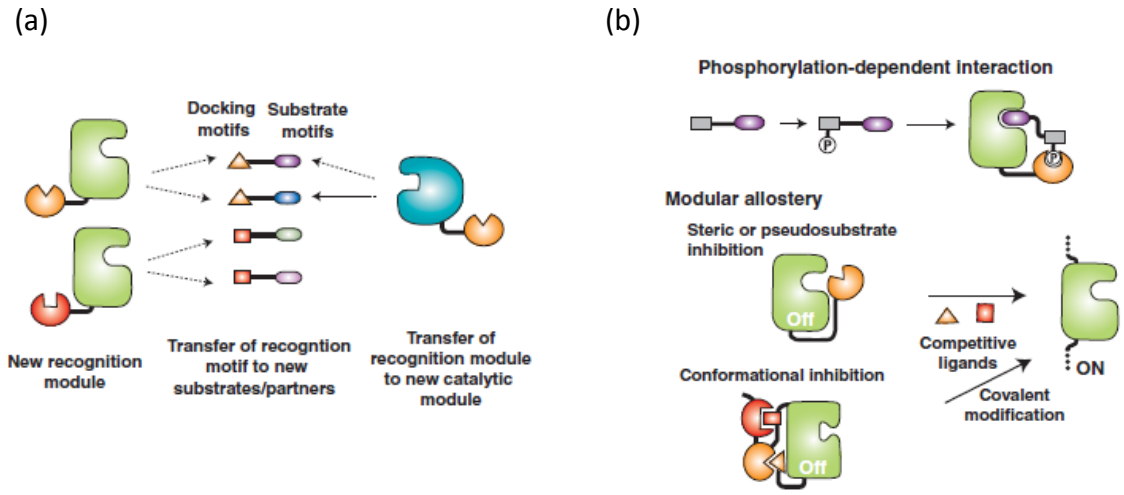

Figure 3. 2 Modular domains interactions. (a) Transferability of modular recognition and catalytic functions: swapping of domains and ligands leads to the formation of new connections betIen proteins which finally gives the possibility of a new set of possible enzyme-substrate interactions and (b) enzyme regulation by modular domains are often used to regulate enzyme activity more directly. These domains can participate in interactions that inhibit catalysis, either by sterically blocking access to the catalytic site or by preferentially stabilizing an inactive conformation of the catalytic domain. These inactive states can then be reversed upon exposure to competing ligands that bind to the domains or by covalent modification of the domains or ligands[64].

3.1.3 Network motifs concept in signaling

In general, the biological networks are highly complex and it is hard to analyze all the molecules involved. It has also been shown that in the biological networks, there are some parts of the network in particular order (arrangement) which plays critical roles in controling the function and the behavior of the cell. These subnetworks are called network motifs. Network motifs are considered as recurring circuits of interactions from which the networks are built. As it is known that to transduce the signal, a cell uses many biological molecules which form a complex signal transduction network, in such complex network, not all the signaling molecules are of equal importance while some of the signaling molecules are crucial in determining the cellular functions and behaviors. The number of such crucial molecules are not so high[71]. To unravel the combination and interaction of these crucial molecules are challenging tasks in network biology. The network motifs are also considered as the circuits which carry out the key information to process the task. In the next step, I would like to introduce the approach (algorithm) which I have used to investigate the signal transduction networks and its behavior.

- **Possible aim for network motifs:**

  It is already known[48,71,72]that in a complex biological network, not all the signaling molecules are of equal importance. While some of the signaling molecules are crucial in determining the cellular functions and behaviors. So, it is of significant importance to analyze the signaling network motifs and unravel their impact on the final cellular response. This network motifs concept may also help in drug targeted therapy approach and might serve as one of the tool in the field of synthetic biology. To find the motifs and their biological importance in signal transduction, it will be a starting point to investigate the topology (in the sense of kinetic parameters) of the evolved signaling networks.

Chapter 4 CONCLUSIONS

1. Based on current results (from section 2.2), I conclude that sustained responses are the generic solution of a SN when the mere detection of the presence of a signal is relevant. This response occurs as soon as the protein-protein interactions are of sufficient strength, either by mutation of the kinetic parameter underlying existing interactions with the network or by recruiting new proteins to the network that generate a sort of bypass by supplying the network with strong interactions. Remarkably, the exact values of the kinetic parameters are irrelevant as soon as a pathway of sufficiently strong interacting proteins is provided. Given the quick evolution of the SNs, I conclude that weak protein-protein interactions serve as a pool to rapidly evolve new pathways, but play only a minor role in modulating the actual responses of a signal transduction network.

   Stronger interactions and addition of new nodes (section 2.8) lead to improved evolved responses. The strength of the signal does not play any role in determining the response nature.
2. From section 2.3 and 2.4 I conclude that irrespective of the protein-protein interaction strength (kinetic parameter), the variation in the concentration of the signaling molecules (receptor, intermediate, and effector molecules) produces transient response when the mere detection of the presence of input signal is relevant. I conclude that the difference between the concentration the receptor, intermediate, and effector molecules also produces transient response although I do not allow the mutation in the concentration of SMs during the evolution optimization.
3. Transient response is controlled by the FBL and the negative cross-interactions between the cascades. If the concentration of the TP is lower than the concentration of the R and ISM, and either FBL or negative cross-talks are present then all the cascade produce consistently transient output response. Irrespective of the concentration of the signaling molecules, FFL and all the positive interactions (cross-talks) between the cascades lead to stable and sustained output response.

Chapter 5 METHODS

5.1 Model

I set up a simplified model to represent a signal transduction pathway allowing two post-translational modifications of similar to the MAPK cascade[5]. In order to transduce the signal, I have included protein-protein interactions, protein phosphorylation and dephosphorylation[73]. Double phosphorylated proteins act as fully activated and single phosphorylated molecules as partially activated molecules. Note, that the term phosphorylation is used for convenience as any other post-translation modification adding a small chemical group, lipid, protein or carbohydrate modifying a protein's spectrum of interaction partners or enzymatic activity are covered by the model.

The interaction between the signaling proteins are set up randomly to create the initial population as well as when adding proteins during evolution. In my current model, I have not classified the proteins of the SN. The kinase or phosphatase function of a protein is determined for each reaction by the matrix A_{ij} (see below). Initially, all the proteins are inactive. One of the initially present inactive signaling molecules is designated as receptor and receives the signal to become activated. The total number of input signals are six and each network is tested for their response to these six different input signal strengths. Once the receptor receives the signal then it can activate other signaling molecules. All the signaling molecules are allowed to phosphorylate or dephosphorylate each other (Figure 2.1) and the final products will be formed depending on the complex. All the reactions in this model are bimolecular, autophosphorylation and homodimer formation are not allowed. Every molecule that becomes partially (single phosphorylated) or full active (double phosphorylated) can interact with any other molecule in any state. These interactions lead to complex formation. The complexes can dissociate without changes to its constituents or upon modifying on of it by means of phosphorylation or dephosphorylation. The interaction of two partially active molecules produces either one of them being fully activated (dual phosphorylated) or inactivated (dephosphorylated) without changing the other reacting partner's state (attributing it an enzymatic role) as shown in Figure 2.1. Which of the possible reactions are realized is determined randomly once at the beginning (with constraints, see next paragraph), thus setting up the network topology.

For feedforward, feedback, and cross-talk in signal transduction study, I have set up a signaling cascade which function in the similar way as MAPK signaling cascade works (Figure S1). This signaling cascade is divided into several different levels of signaling such as receptor level (represented as R), intracellular signaling level (as ISM), and the target level represented as TP (the target proteins are those proteins which communicate the information to the nucleus in the form of the output response. Then, I have added different kinds of loops and the cross-interactions (cross-talks) between two signaling cascades at different levels of signaling. In the next step, I have created mass-action kinetic model by using the ordinary differential equations (ODEs) for all the molecules including the complexes formed as result of chemical reaction

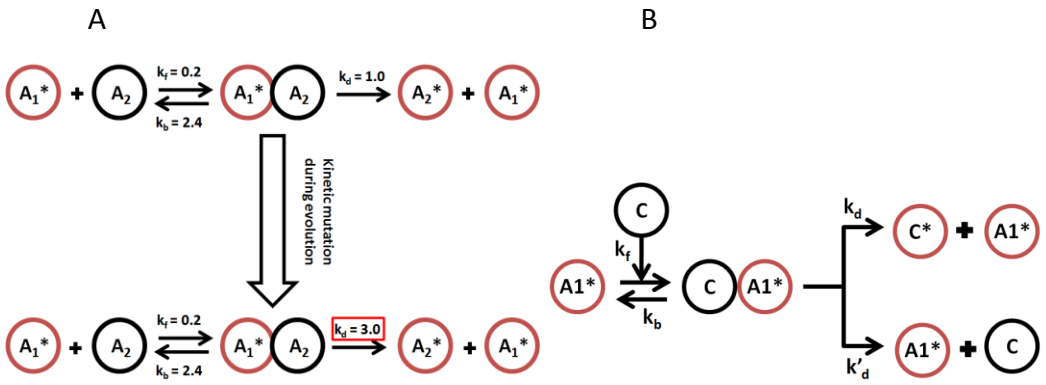

Figure 5.1 Interaction strength and addition of new nodes. (A) Mutation of kinetic parameter. A1* represents the active form of the protein A1, A2 another inactive protein molecule, A1*A2 is the complex formed during the reaction between A1* and A2. A2* is the active form of A2. k_f, k_b, and $k_d$are the rates (interaction strength) of the reactions. A mutation of the reaction alters any of the rates, e.g., k_d = 1.0 (top) adopts the new value k_d = 3.0. (B) Addition of new node in the minimal model. An inactive protein C is added, that can either become activated (C*) by A1* (k_d) or is inactivating the active protein A1* (k'_d).

In addition, one randomly selected double phosphorylated protein, different from the receptor node, is designated the output node. It represents the molecule which will eventually induce the cellular response.

5.2Reaction details of the model

Here, I have shown the details of the possible reaction during signal transduction process. Figure 5.2 shows the possible reaction for three nodes network while Figure 5.3 a and b show additional reactions taken place after the addition of fourth node in the three node network (minimal model).

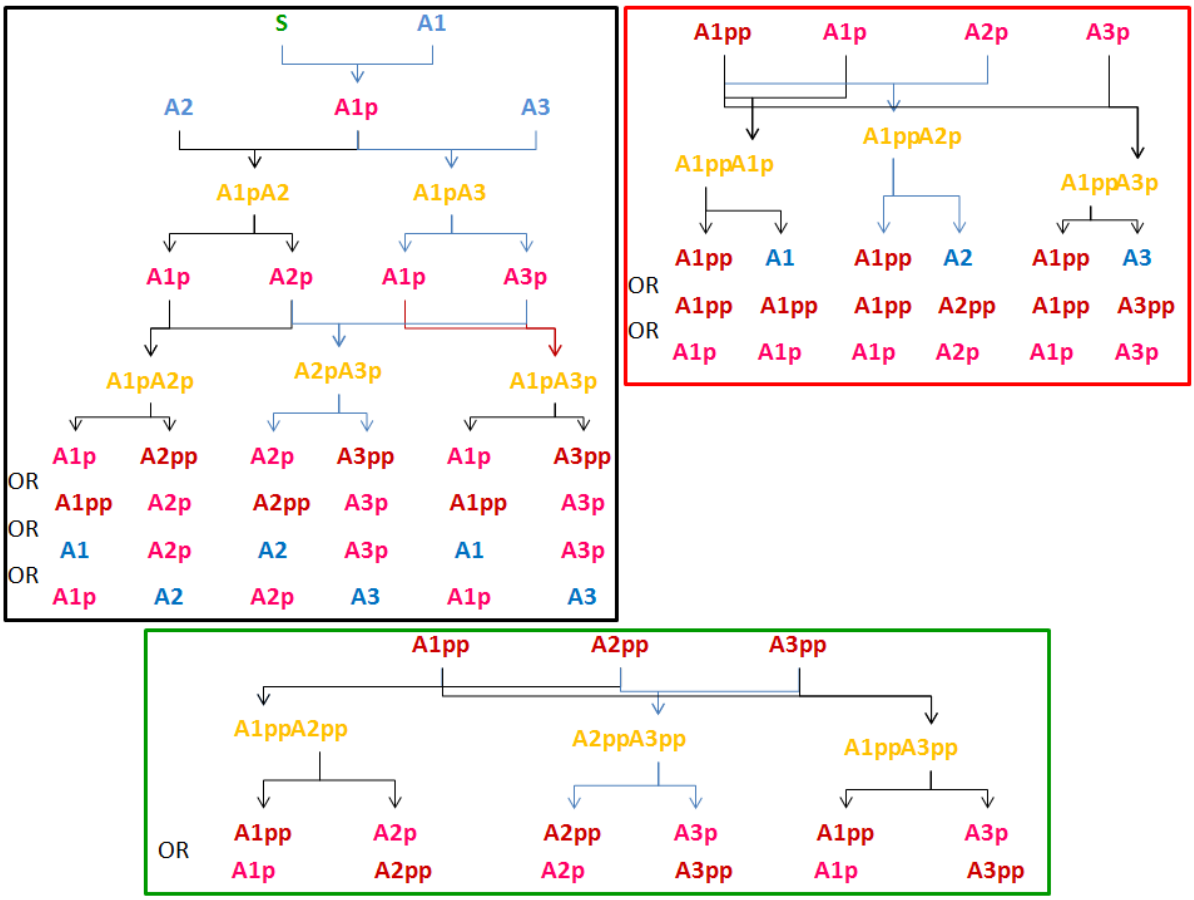

Figure 5.2 List of possible reactions in the minimal model with three proteins. S represents the input signal, A1, A2, and A3 denote inactive signaling proteins, their partially active (single phosphorylated) forms are A1p, A2p, and A3p, respectively. The fully active forms are A1pp, A2pp, and A3pp.

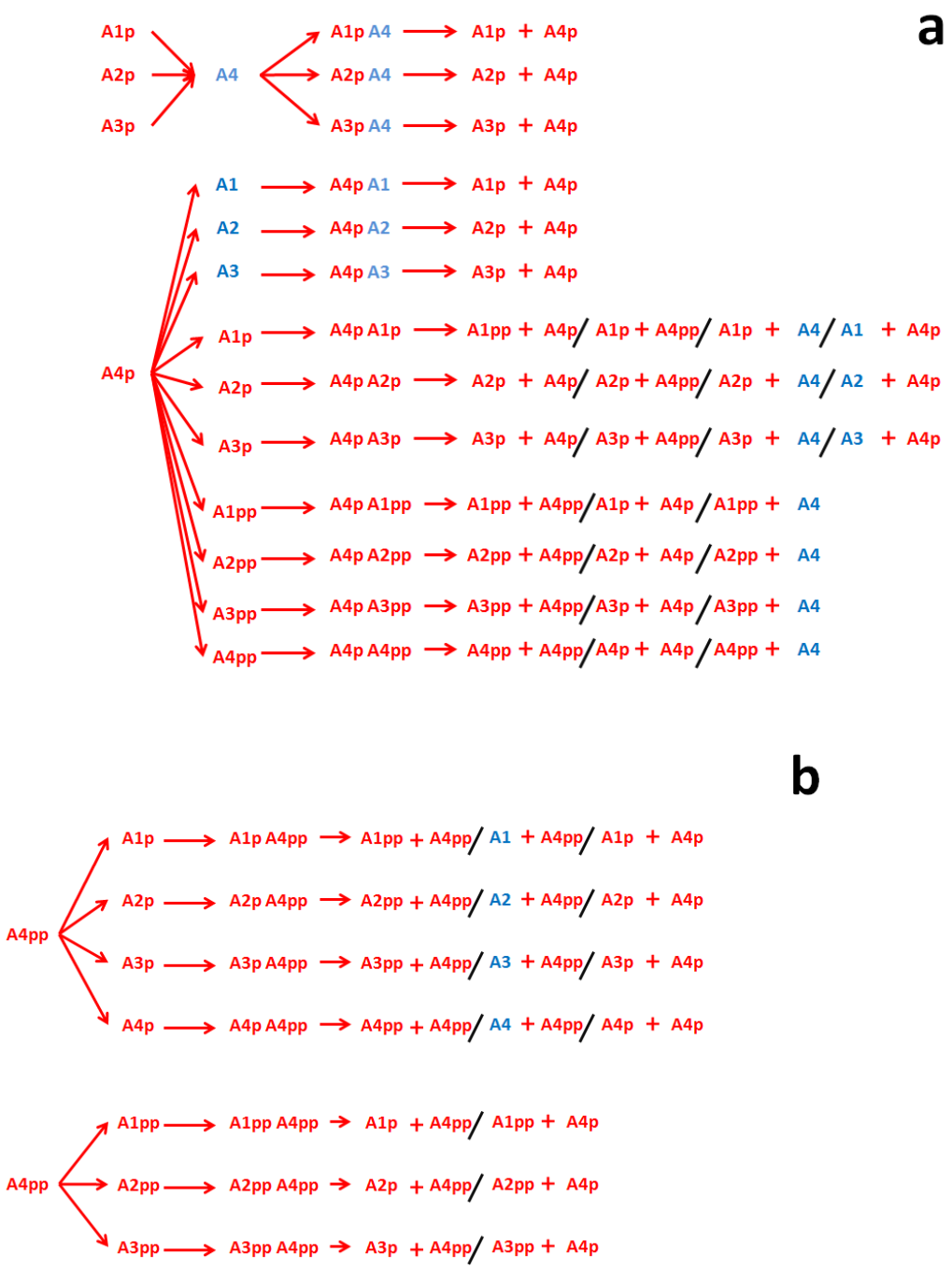

Figure 5.3 List of possible reactions after the addition of a new node designated as A4 in the minimal model. A1, A2, A3, and A4 denote the inactive signaling proteins, their partially active (single phophorylated) forms are A1p, A2p, A3p, and A4p, respectively and their fully active forms are A1pp, A2pp, A3pp, and A4pp, respectively.

5.3 Generalized mass-action kinetics equation

A network consists of the above mentioned signal transduction pathway where both inactive and active proteins and complexes are represented as nodes. The interaction matrix (A_{ij}) between all these molecules including complexes are represented as +1 (production/generation), -1 (degradation/dissociation), and 0 (no interaction). The entries of A_{ij} are chosen once for a network under the constraints that the total amount of each protein is conserved and the SN generated does have a stable inactive state.

The entries A_{ij} generate the index/indices for the reactant(s) $x_{p(r)}$ for the reaction r. Each arc encoded by the interaction matrix is associated with a weight that represents the kinetic parameters with which production or degradation takes place. The dynamics x_i of the node i is governed by the equation:

$$\frac{dx_i}{dt} = \sum_{r=1}^{r_{tot}} A_{ir} k_r \prod \left[x_{p(r)}\right] (1)$$

k_r denotes the kinetic parameter of the reaction number r. Note, that I chose k to be dimensionless in the sense that the time is scaled appropriately and the concentrations are normalized such that the numeric values of first- (k_2, k_3 in Figure5.1) and second-order (k_1 in Figure5.1) reactions approach a similar range.

The fitness of a SN was tested by calculating its response to n_s = 6 different signals. For every signal n, the dynamics of the pre-selected output node is tested whether it exceeds a threshold f. This threshold ($f = \frac{1}{10} th$ of the initial concentration level) is defined to be the relative fraction of the double phosphorylated protein to the total amount of the protein. If the output node crosses the threshold f at any point during the dynamics the network gains a fitness contribution $F_{factor}(n) = 1$. The normalized fitness F_{norm} is calculated as the average fitness contribution for all signals which are weighted equally:

$$F_{norm} = \frac{\sum_{n=1}^{n_s} F_{factor\ (n)}}{n_s} (2)$$

Hence, the maximum fitness of a network will be 1 when it detects all signals or 0 when there is no above-threshold response to any of the signals.

5.4 Algorithm

I have applied an evolutionary algorithm [20]to evolve the networks (Figure5.4). Before starting evolution, I create a set of diverse networks with the same randomly generated interaction matrix for three proteins with three states (un-, mono-, and dual-phosphorylated) and different randomly selected kineticparameters (Equation 1). The evolution of the networks is either controlled by mutation of the kinetic parameters or the addition of new nodes. In the latter case kinetic parameters are randomly selected once at the generation of the initial population and once for every single newly added protein. The kinetic parameters are generated randomly initially in the range 0.001 to 1. The total number of the networks is N = 200. For each network, F_{norm} is computed. In each generation, I have calculated the mean of the fitness (F_{norm}) in each genration.I perform elite selection of $f = \frac{1}{4}th$ of the population. The successful networks are mutated by changing the kinetic parameters (k_r) or adding new proteins as explained above. The subsequent generation is then populated by four copies of the successful networks keeping the number N of networks identical in each generation. I evolve the population of networks for 200 generations. Systems of ordinary differential equations were formulated and solved with MATLAB 7.9.0.

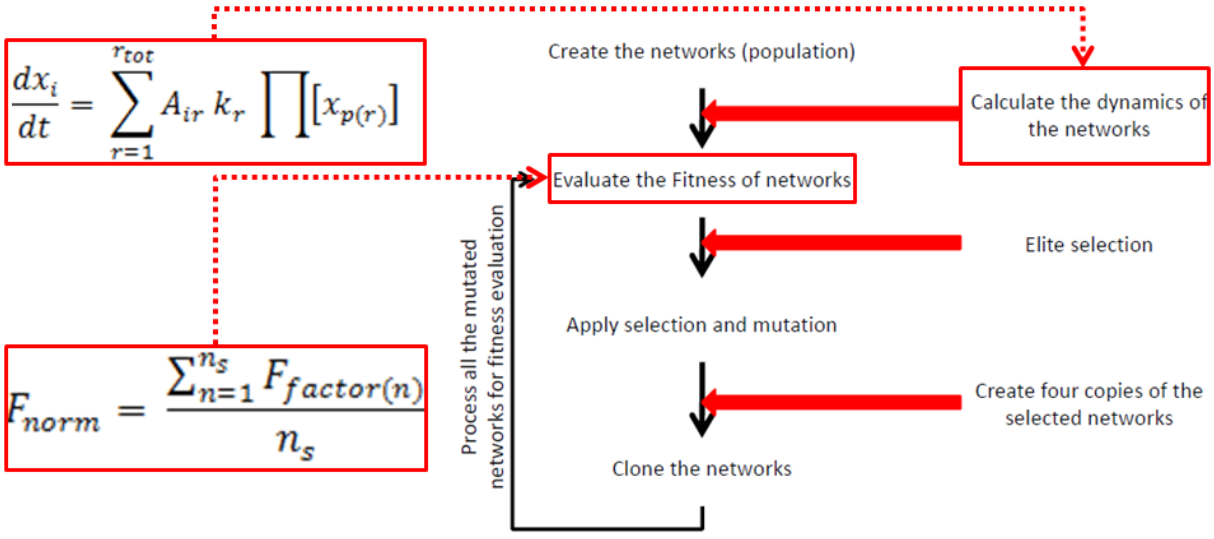

Figure 5.4 Scheme of the evolutionary algorithm. In order to generate genetic diversity, a set of 200 networks is created with diverse sets of kinetic parameters. In each iteration the dynamics of all networks is calculated for the complete set of input signals. Based on dynamics, the fitness is calculated. Based on the fitness scores, the successful network are selected (elite selection). For each selected network mutations are either applied to the kinetic parameters or the topology of the network by adding new proteins. Each selected network gives rise to an equal number of clones such that the population contains again 200 networks.

5.5 Methods for investigating the roles of the cross-talk of signal transduction pathways

For investigating the roles of different possible cross-talks between the signal transduction pathways, the method was exactly the same as discussed in the above mentioned sections 5.1, 5.2, 5.3, and 5.4. I have selected the signaling molecules from both the pathways for interaction and then created a set of signaling pathways (similar and dissimilar pathways) with different sets of kinetic parameter values. The networks have been evolved in the similar way as discussed in the previous section.

As mentioned in section 1.4, in biological systems different kinds of cross-talk have been found in signaling systems. Those different cross-talk systems have been represented in generalized forms. According to Papin J A. and Palsson B O. (2004)[50], cross-talk is the nonnegative linear combination of the signaling pathways. The pair-wise combination of pathways is the simplest form of cross-talk. They have classified cross-talks in SNs different categories based on the extreme pathways.

In my thesis work, I have created two similar kind of pathways (Figures 5.5 A and B) which can detect both similar and dissimilar input signals and the pathways can cross interact with each other through any of the molecules. The interaction may be activatory or inhibitory in nature. Based on the nature of interaction response 1 and 2 can be generated. In my thesis work, I have shown only four of the possible cross-talk and their results. All other types of possible cross-talk will be carried out in the next step as a future perspective.

For my thesis work, I have investigated four possible cross-talks between two similar kind of pathways. These cross-talks are either activatory or inhibitory in nature which means one of the pathway can either activate one of the molecule of another pathway or inhibit the pathway by targeted blocking of one of the molecule. The molecules selected to either activate or inhibit was random.

5.6 Experimental details

Dr. Tina M. Schnöder performed western blot experiment and analysis to verify our findings for the effect of concentration of the SMs on the cellular response where I conclude that difference in the concentration of signaling molecules leads to the transient cellular response.

Cell culture: Ba/F3 cells were cultured in RPMI1640 medium (PAA) supplemented with 10% fetal bovine serum (PAA) and with 1 U/ml of human recombinant Erythropoietin (EPO) (Janssen-Cilag) in a humid atmosphere of 5% CO2 at 37 °C.

Immunoblotting: Ba/F3 cells expressing EpoR and wildtype JAK2 were washed twice with PBS and starved for 4 h in serum-reduced (0.5%) medium at a density of 1x106/ml.. Cells were re-stimulated with different EPO concentrations (0, 0.1, 0.5, 1, 2 U/ml) for 5, 10 or 30 min and lysed as described previously[74]. The following antibodies were purchased from Cell Signaling and used in 1:1000 dilution: p-p44/42 MAPK (9106), p44/42 MAPK (9102). GAPDH antibody (H86504M, 1:5000) was purchased from Meridian Life Sciences.

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RESUME

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EXPERIENCE

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EDUCATION

- 08/2010—06/2013 **Ph.D.** in Systems Biology, Otto-von-Guericke University, Magdeburg, Germany
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- 07/2004—07/2006**M.Sc.**in Bioinformatics, JamiaMilliaIslamia, New Delhi, India
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COMPUTATIONAL SKILLS

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WETLAB SKILLS

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AWARDS

- 05/2012 Travel Grant Fellowship Award, Integrative Network biology 2012: Network Medicine, Helsingoer, Denmark
- 09/2008—08/2010 Jacobs University Fellowship for study Mol. Life Sci. (Comp. Biol.) at Jacobs University Bremen, Germany
- 02/2008 Travel Grant Fellowship Award, International Biomedical Modeling School and Workshop, Bangalore, India

PUBLICATIONS

- Mobashir M, Schraven B, and Beyer T (2012) Simulated Evolution of Signal Transduction Networks. PLOS ONE 7(12): e50905.

- Mobashir M, Schnoeder TM, Fischer T, Schraven B, and Beyer T (2013) Modulation of Cellular Response: An Evolutionary Approach (In Rivision: PLOS ONE).
- Mobashir M, Madhusudhan T, Isermann B, Beyer T and Schraven B (2013) Negative Interactions and Feedback Regulations are Required for Transient Cellular Response(Under Review: PNAS).
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CONFERENCE/SYMPOSIUM

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REFERENCES

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