PROJECT REPORT

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Reconstruction and Modeling of MAP Kinase signaling pathway

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CERTIFICATE

This is to certify that the project entitled "Reconstruction and modeling of MAP Kinase signaling pathway" is a bonafide work carried out by **Sonia Chothani,** Second Year B.Tech in Biotechnology, IITMadras, Chennai at the Centre for Cellular & Molecular Biology, Hyderabad under my guidance, during the period of 12th May 2010 to 12th July 2010.

(Dr. Ram Rup Sarkar)

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Sonia Chothani

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Introduction

Biological Signaling Networks

Network study is necessary to obtain an in depth insight into the molecular mechanisms of an organism. It also helps in differentiating between mechanisms of different organisms. Cell signaling networks are part of complex system of communication that governs basic cellular activities and coordinates cellular actions. Signaling pathway is a mechanism that converts extracellular signal to a cell into a specific cellular response. There could be various kinds of signals/stimulus like cytokines, growth factors, hormones or even environmental stimuli like stress, odor, light etc. These signals are transmitted to the cell by binding to receptors thereby bringing about a conformational change in the receptor. After which this receptor further transmits the signal by binding or phosphorylation (and other means) with the help of molecules inside the cell (secondary messengers). This is how the signal is transmitted and then leads to different cellular responses like cell proliferation, apoptosis, etc. The ability of cells to perceive and correctly respond to their microenvironment is the basis of development, tissue repair, and immunity as well as normal tissue homeostasis. Errors in cellular information processing are responsible for diseases such as cancer, autoimmunity, and diabetes. Signaling networks are the perceptual components of a cell and are therefore responsible for observing current conditions and making decisions about the appropriate use of resources — ultimately by regulating cellular behavior [1].

These networks allow living organisms to issue an integrated response to current conditions and make limited predictions about future environmental changes. Analysis of these networks contributes to a deeper understanding of network-wide interdependencies, causal relationships, and basic functional capabilities. While the structural analysis of metabolic networks is a well established field, similar methodologies have been scarcely applied to signaling and regulatory networks. By understanding cell signaling, diseases may be treated effectively and, theoretically, artificial tissues may be yielded. Hence it is of utmost importance to study such signaling networks to understand the regulation and other cellular responses.

Reconstruction of Signaling Pathways

Signaling pathways are available on various online databases like KEGG (Kyoto Encyclopedia of Genes and Genomes), Protein Lounge, CST (Cell Signaling Technology Pathway database), Panther (Protein ANalysis THrough Evolutionary Relationships), PID (NCI-Nature Pathway Interaction Database), etc, [8 -12] but there are incomplete and inconsistent information available in all these databases. Further, not all the databases provide the detail of the reactions, molecules and enzymes involved in the pathways. Integrating information from different databases and existing literatures to reconstruct the major pathways and thereby linking these pathways to form a larger network to study different effects (e.g. gene deletion, mutation, perturbation etc.), is in itself a challenging problem. Hence while reconstruction one needs to compare as many databases as possible and simultaneously cross-check with published literature. Moreover, in these networks, links are based on pre-established biomolecular interactions; significant experimental characterization is thus needed to reconstruct biochemical reaction networks in human cells. Hence a network reconstruction includes a chemically accurate representation of all of the biochemical events that are occurring within a defined signaling network, and incorporates the interconnectivity and functional relationships that are inferred from experimental data [2].

For signaling networks it is important to know almost all possible interactions of a molecule because it might be activating or inhibiting some other important pathways, and regulate the cellular responses. Hence an attempt should be made to make a comprehensive pathway with all possible interactions. After reconstruction of the pathway, a systematic verification with published research papers should be made in order to confirm absence of inconsistencies.

Human Specific - Mitogen Activated Protein Kinase (MAPK) pathway

The Mitogen Activated Protein Kinases (MAPK) are serine/threonine specific kinases. Mammals express at least four distinctly regulated groups of MAPKs, Extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38a/b/g/d) and ERK5 that are activated by specific MAPKKs: MEK1/2 for ERK1/2, MKK3/6 for thep38, MKK4/7 (JNKK1/2) for the JNKs, and MEK5 for ERK5. Each MAPKK, however, can be activated by more than one MAPKKK, increasing the complexity and diversity of MAPK signaling. Presumably each MAPKKK confers responsiveness to distinct stimuli. For example, activation of ERK1/2 by growth factors depends on the MAPKKK c-Raf, but other MAPKKKs may activate ERK1/2 in response to pro-inflammatory stimuli [3].

Importance of MAPK signaling pathway

MAPK cascade is a highly conserved module that is involved in various cellular functions, including cell proliferation, differentiation and migration. This pathway occurs in almost all kinds of cells and mutations in the pathway may lead to harmful abnormal responses, like uncontrolled proliferation (Cancer). Hence a careful study of this pathway will help in understanding the progression of the disease.

Mathematical Modeling and Analysis Approach

Simulation and modeling is becoming a standard approach to understand complex biochemical processes and large networks, since it is more streamlined and hence less time-consuming than the experimental approach. Recent developments in integrative approaches, mathematical and computational methods, have been found to be indispensable tools in understanding such complex systems. The constraint-based stoichiometric analysis technique Flux balance analysis (FBA), has been applied to study the metabolic capabilities of several systems based on the mass balance

constraint [24], and has provided useful insights into cellular behavior, including response to perturbations such as gene deletions. On the other hand, Logical Steady State Analysis (LSSA) is a newly introduced approach, which facilitates a structural analysis of signaling and regulatory networks with special focus on functional aspects [6]. LSS analysis has a number of applications for studying functional aspects in cellular interaction networks, specifically signaling pathways, for example- imposing different patterns of signals one may check which molecules become activated or inhibited in the intermediate and, in particular, output layer. Further, the changes in signal flows and input-output behavior occurring in a manipulated or malfunctioned network can be studied by removing or adding elements or by fixing the states of certain species in the network. Knowledge of the set of signaling paths and feedback loops facilitates the computation of intervention strategies.

Objective of the work

The major objective of this work is to reconstruct a comprehensive MAPK signaling pathway from different database and literature. We aim to model the signaling events occurring in this pathway to identify the key molecules and interactions of the complex pathway. The Approach which we used for modeling and analysis is Logical Steady State Analysis (LSSA), which enables studies on the logical processing of signals and the identification of optimal intervention points (targets) in cellular networks. We used the software CellNetAnalyzer (CNA) for this purpose [25]. Further, a systematic perturbation study analysis to identify alternative pathways and optimal intervention points was carried out.

Methods

Literature and Database Study

Signaling maps are constructed from annotated genome sequence data, biochemical literature, bioinformatics analysis, and human-specific information. These pathways are available on various databases. The following databases have been consulted for reconstructing MAPK pathway.

1) **KEGG PATHWAY database:** It consists of graphical representations of cellular processes, such as metabolism, membrane transport, signal transduction and cell cycle. One of the most organized databases consists of 2706 entries for pathway diagrams from 143 manually drawn diagrams [7, 8].
2) **Protein Lounge:** The Pathway Database is the largest collection of signaling transduction and metabolic pathways. All pathways include extensive reviews and detailed protein information. All proteins in each pathway are linked to detailed information about them. The pathway database is a foundation for understanding the mechanism of cellular signaling and an essential tool for any researcher [9].
3) **Panther Database**: PANTHER Pathway consists of over 165, primarily signaling, pathways, each with subfamilies and protein sequences mapped to individual pathway components. Pathways are drawn using Cell Designer software, capturing molecular level events in both signaling and metabolic pathways, and can be exported in SBML format [10].
4) **NCI-Pathway Interaction database**: The Pathway Interaction Database is a highly-structured, curated collection of information about known biomolecular interactions and key cellular processes assembled into signaling pathways. It consists of 108 human pathways and 7086 interactions [11].
5) **Cell Signaling Technology pathway database:** The revised and updated diagrams have been assembled by Cell Signaling Technology (CST) scientists and outside experts to provide succinct and current overviews of selected signaling pathways [12].

6) **BioModels:** BioModels Database is a data resource that allows biologists to store, search and retrieve published mathematical models of biological interests [13].
7) **HCPIN (Human Cancer Protein Interaction Network:** It is based on 7 Pathways: apoptosis, cell-cycle, JAK, MAPK, PI3K, TGF, TLR and has 2977 Proteins and9784 Interactions [14].
8) **DOCQS (Database Of Quantitative Cellular Signaling):** The Database of Quantitative Cellular Signaling is a repository of models of signaling pathways. It includes reaction schemes, concentrations, rate constants, as well as annotations on the models [15].
9) **Reactome:** REACTOME is a free, online, open-source, curated pathway database encompassing many areas of human biology Pathway data can be exported in SBML and BioPAX formats [16].
10) **InnateDB:** It is a publicly available database of the genes, proteins, experimentally-verified interactions and signaling pathways involved in the innate immune response of humans and mice to microbial infection [17].
11) **SigPath:** SigPath is an information system designed to support quantitative studies on the signaling pathways and networks of the cell [18].
12) **Wiki Pathways:** WikiPathways was established to facilitate the contribution and maintenance of pathway information by the biology community. WikiPathways is an open, collaborative platform dedicated to the curation of biological pathways [19].
13) **NetPath**:20 pathways are freely available in BioPAX, PSI-MI and SBML formats at this website [20].

We manually constructed a comprehensive pathway map for MAPK signaling cascades based on the above databases and published scientific papers [26 - 32]. MAP Kinase signaling pathway from the KEGG Database was taken as the basic model (179 proteins and 110 reactions), additions and modifications were made to it on the basis of the molecular interactions documented in 70 published papers (see References for MAPK signaling pathway) and comparing it with other databases. The reconstructed

pathway comprises of approximately 297 proteins and 161 reactions (see Figure 8 in Result Section).

Software*:*

CellNetAnalyzer (CNA) was used for modeling and analysis of this pathway. *The CellNetAnalyzer (CNA)* is a Matlab supported software and provides a comprehensive and user-friendly environment for structural and functional analysis of biochemical networks within a graphical user interface. The abstract network model (.jpg/.bmp/.tiff format) is linked with network graphics leading to an interactive signal analysis map, which allows user input and display of calculated results within network visualization. 'Specie' is a term defined by CNA as 'an entity that takes part in reactions' and it is used to distinguish different states that are caused by enzyme modifications, association, dissociation and translocation.

Before composing our network, we first standardized the software using a known network and published results. We took a simple example, *Signaling toy network* (see Figure 1) for standardization of this software.

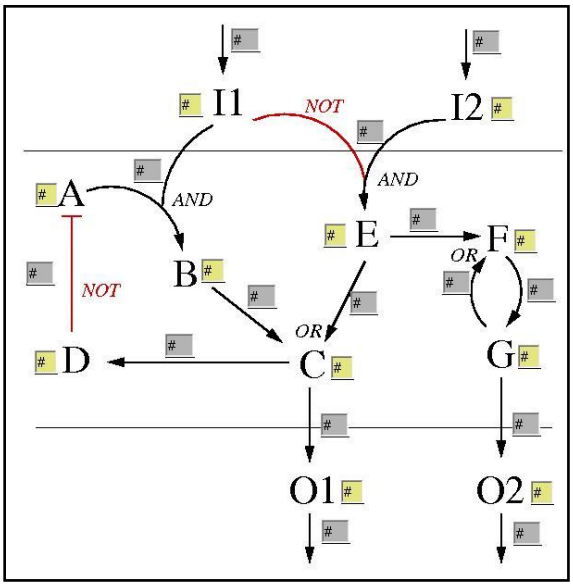

Figure 1: Signaling Toy Network composed in CellNetAnalyzer

The Network has 11 Species: I1, I2, A, B, C, D, E, F, G, O1, O2; and 15 Interactions:

=I1	O2=	B = C	E = C	G = F
=I2	I1 + A = B	C = D	E = F	C = O1
O1=	!I1 + I2 = E	!D = A	F = G	G = O2

The pathway was then simulated in CNA and the dependency matrix (see Figure 2) was compared with results given in published literature [6].

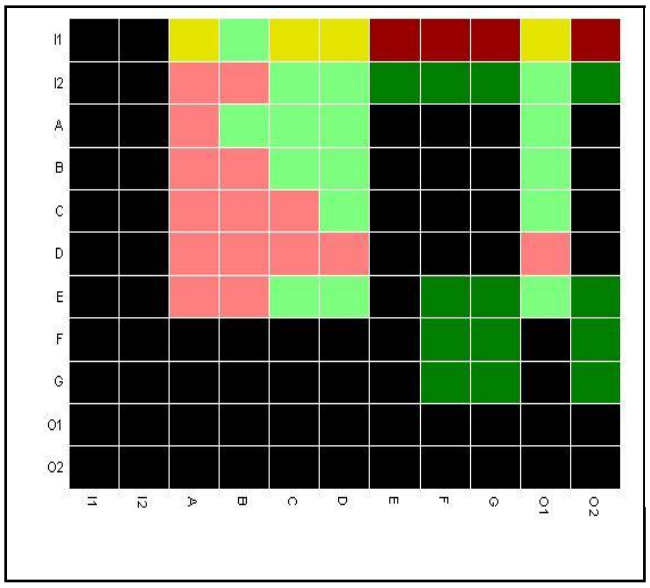

Figure 2: Dependency Matrix of Signaling Toy Network.

Color Index:
(i) Dark Green: x is a total activator of y
(ii) Dark Red: x is a total Inhibitor of y
(iii) Light Green: x is a (non-total) activator of y
(iv) Light Red: x is a (non-total) inhibitor of y
(v)Yellow: x is an ambivalent (both activate and inhibits) factor for y
(vi) Black: x does not have any influence on y

Next, we proceed to model the reconstructed MAPK signaling pathway.

Network Composition in CNA

First, we define each species in the network, then all the interactions. The interactions are defined by Logical Equations consisting of AND, OR, NOT operators. For example, RAS is being independently activated by Grb2-SOS and PKC and is inhibited by Gap1m (shown in Figure 3(a)). This reaction is such that in the presence of Gap1m RAS gets inhibited irrespective of anything else whereas when gap1m is absent RAS depends on whether either of PKC and Grb2-SOS is present.

Now for defining the logical equation for these interactions we have to form a logical equation for the sink (specie having no successor), here RAS. Firstly we try to find the relation between the two activators. Writing the truth table (see Table 1) for the

two activators we observe that Activators = Grb2-SOS + PKC. Now writing the truth table (see Table 2) for activators and the inhibitor, we observe RAS=Activators.(!Gap1m)

Figure 3: a) Activation and inhibition of RASby other species; **b)** Activation of PI3K by RAS and other species (**Grb2-SOS:** Growth factor receptor-bound protein – Son of Sevenless (GEF) complex; **PKC:** Protein Kinase C; **Gap1m:** RAS GTP-ase activating protein (GTP hydrolysis); **Ras**: GTP-ases; **PI3K:** Phosphoinositide 3 kinase)

These logical equations are stored by CNA in the form of an interaction hyper-graph. An interaction graph is a matrix between the reactions and species taking individual interactions, and an Interaction hyper-graph is a matrix between the combined reactions (as defined by our logical equations). The logical steady state of each molecule is tabulated (Table 1 and 2). When one of the activators is present Ras should be activated hence the red marked values are incorrect so the logical equation should have an OR operator (Table 1). When both activators and inhibitor are absent then Ras cannot be activated so the 1st row red marked value is incorrect, hence the logical equation should have an AND operator (Table 2).

Table 1: Truth table for activators

Grb2-SOS	**PKC**	**OR**	**AND**
0	0	0	0
0	1	1	0
1	0	1	0
1	1	1	1

Table 2: Truth table for activators and inhibitor

Activators	**!Gap1m**	**OR**	**AND**
0	0→ 1	1	0
0	1→0	0	0
1	0→1	1	1
1	1→0	1	0

Again for RAS (shown in Figure 3(b)) we write the Interaction graph and the Interaction hyper-graph (see Figure 4).

Interaction Graph

1(+)	2(+)	3(-)	4(+)	
-1	0	0	0	Grb2-SOS
0	-1	0	0	PKC
0	0	1	0	PI3K
0	0	0	-1	Gap1m
1	1	-1	1	RAS

Interaction Hyper-graph

(1+2)&!4	3	
-1	0	Grb2-SOS
-1	0	PKC
0	1	PI3K
-1	0	Gap1m
1	-1	RAS

Figure 4: L-R Interaction graph, Interaction Hyper-graph

Validation of Model

After defining the complete network we validated our model. We simulate the network to compare it with published experimental results so as to check the plausibility and consistency of the simulations

For Example, PTEN is a tumor suppressor gene. According to the experiments on cancer cell lines it was detected that PTEN undergoes homozygous deletions, frameshift or nonsense mutations. Thus there is a Loss of heterozygosity (LOH) which leads to inactivation of the normal function of PTEN [21]. So we can say that in cancer cell lines PTEN is in its mutated form (inactive) and in normal cell lines we have non-mutated PTEN (active) which suppresses the tumor.

Simulation analysis:

We did a simulation analysis to verify our model with the experimental results. So we considered one of the oncogenes (Epidermal Growth Factor) keeping it ON (state = 1) and fixed certain states for every input molecules (See Table 3) and checked the response on varying PTEN activity.

Table 3: Fixed Values for Input signals

Input Molecule	**Value**	**Input Molecule**	**Value**	**Input Molecule**	**Value**
EGF	1	NF1	0	Ask2	1
FGF	0	P120GAP	0	PP2CA	0
PDGF	0	G12	0	PP2CA	0
IL-1	1	PTP	0	PP5	0
TNF-alpha	1	MKP	0	JIP1/2	1
FASL	1	Mos	1	Evil	0
LPS	1	RacGDP	1	Bcl2-Bxl	0
TGFB	1	PIP3	1	Sodd	0
Bax/Bak	1	TpI2/Cot	1	WNK	0
MP1	1	Muk	1	Bim	0
NGF	0	BDNF	0	NT3/4	0

When PTEN was kept OFF; State = 0 (inactive form-mutated form) we observed that transcription factors that lead to uncontrolled proliferation were activated and only one transcription factor which leads to apoptosis was activated hence leading to tumor progression (see Figure 5). Whereas when PTEN is kept ON; State = 1 (normal active form - not mutated) the transcription factors which lead to uncontrolled proliferation are inhibited and only few which lead to both apoptosis and proliferation are activated thus leading to a normal cell cycle (see Figure 6).

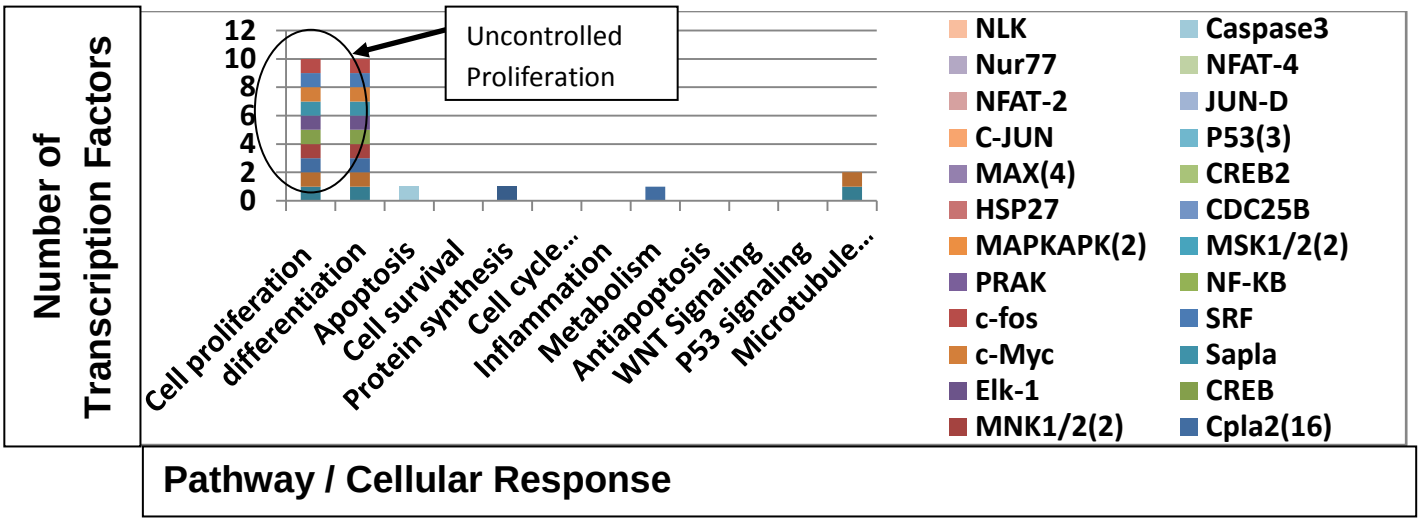

Figure 5: Expression of different transcription factors for different cellular processes when PTEN is kept OFF. We observe that 10 Transcription factors that lead to proliferation are activated whereas only 1 which leads to apoptosis is activated, hence uncontrolled proliferation observed.

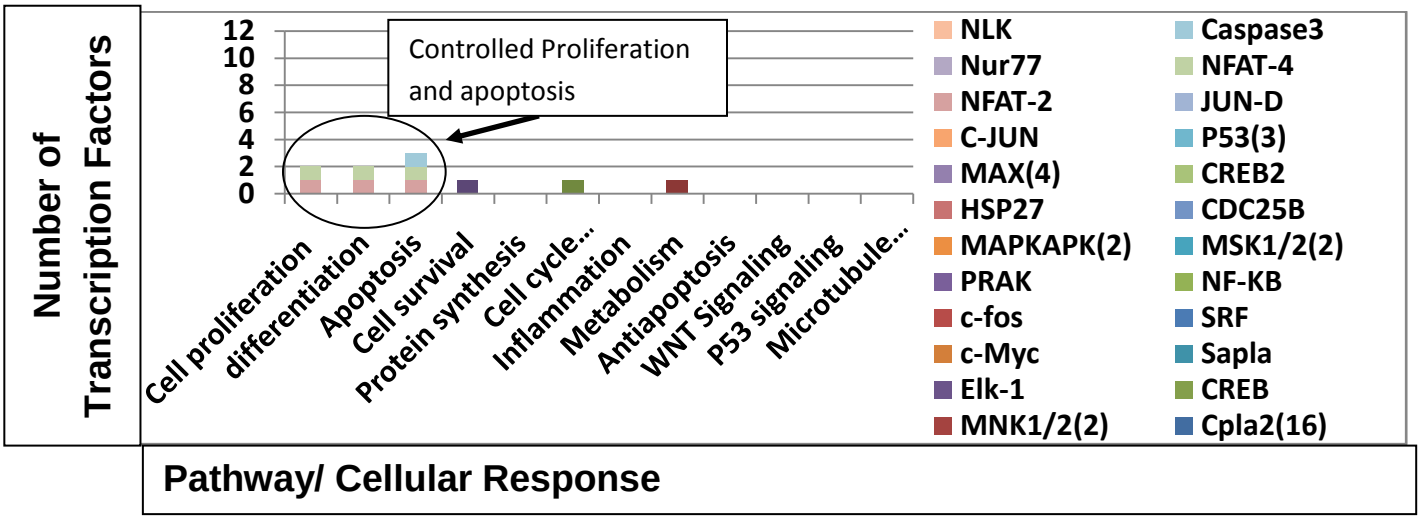

Figure 6: Expression of different transcription factors for different cellular processes when PTEN is ON. We observe that 2 Transcription factors that lead to proliferation are activated and 3 transcription factors that lead to apoptosis are activated, hence a controlled proliferation and apoptosis is observed.

Results & Discussions

We observed a lot of inconsistency and incomplete data in various databases for MAPK pathway. Like in Protein Lounge the Ras-PI3K pathway has been reported whereas it is not shown in KEGG, but this pathway according to literature occurs and in fact is important because it directly affects cell cycle responses [30].

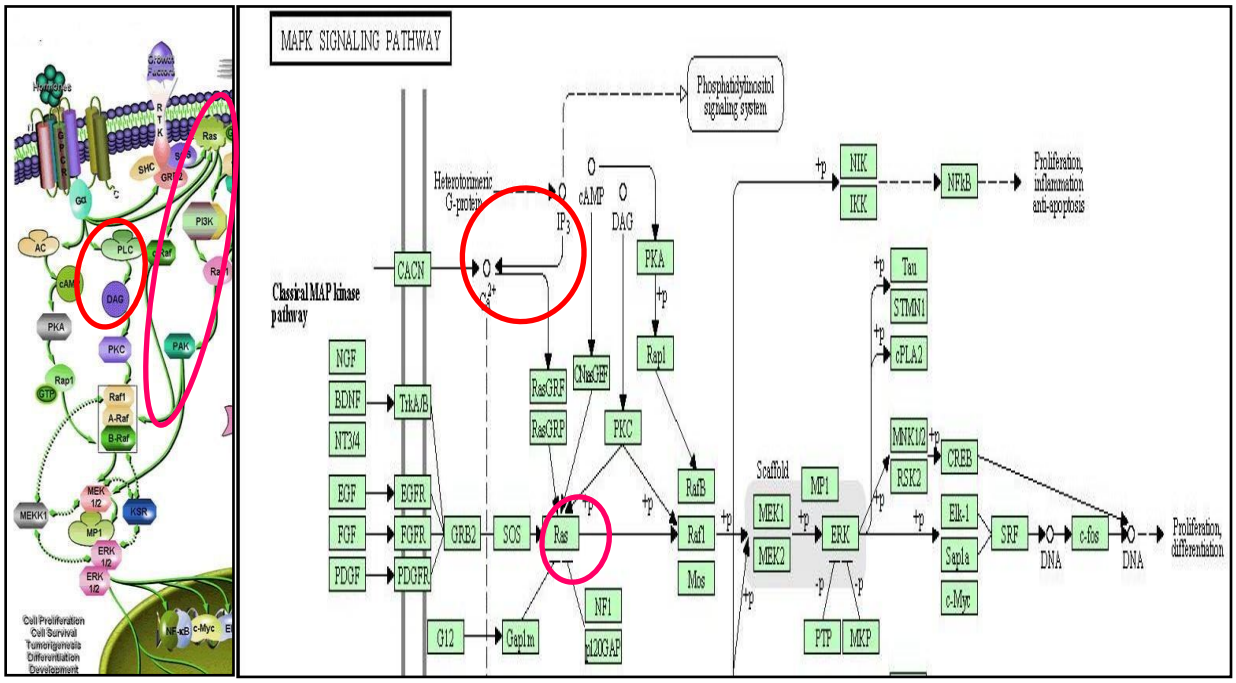

Figure 7: Classical MAPK signaling pathway from L-R: Protein Lounge Database, KEGG Database. Pink Oval: Ras → PI3K pathway absent in KEGG; Red Oval: IP3 & Calcium signaling absent in Protein Lounge

The number of molecules and number of interactions reported in some major database with varying results are given in Table 4. This justifies the importance of reconstruction of the pathway for any analysis.

Table 4: Comparison of No. of Species and Interactions in MAPK signaling pathway between various databases

Database	No. of Species	No. of Interactions
KEGG	179	110
Protein Lounge	120	75
Cell Signaling	155(90+35+30)	120(60+25+35)
Panther	36	44
BioModels	34(26+8)	30(20+10)
NPID	80(45+35)	95(55+40)

The complete MAPK signaling pathway consisting of ERK, JNK, P38 and ERK5 sub-pathways was reconstructed (see Figure 8). It consists of 297 species and 161 interactions after reconstruction.

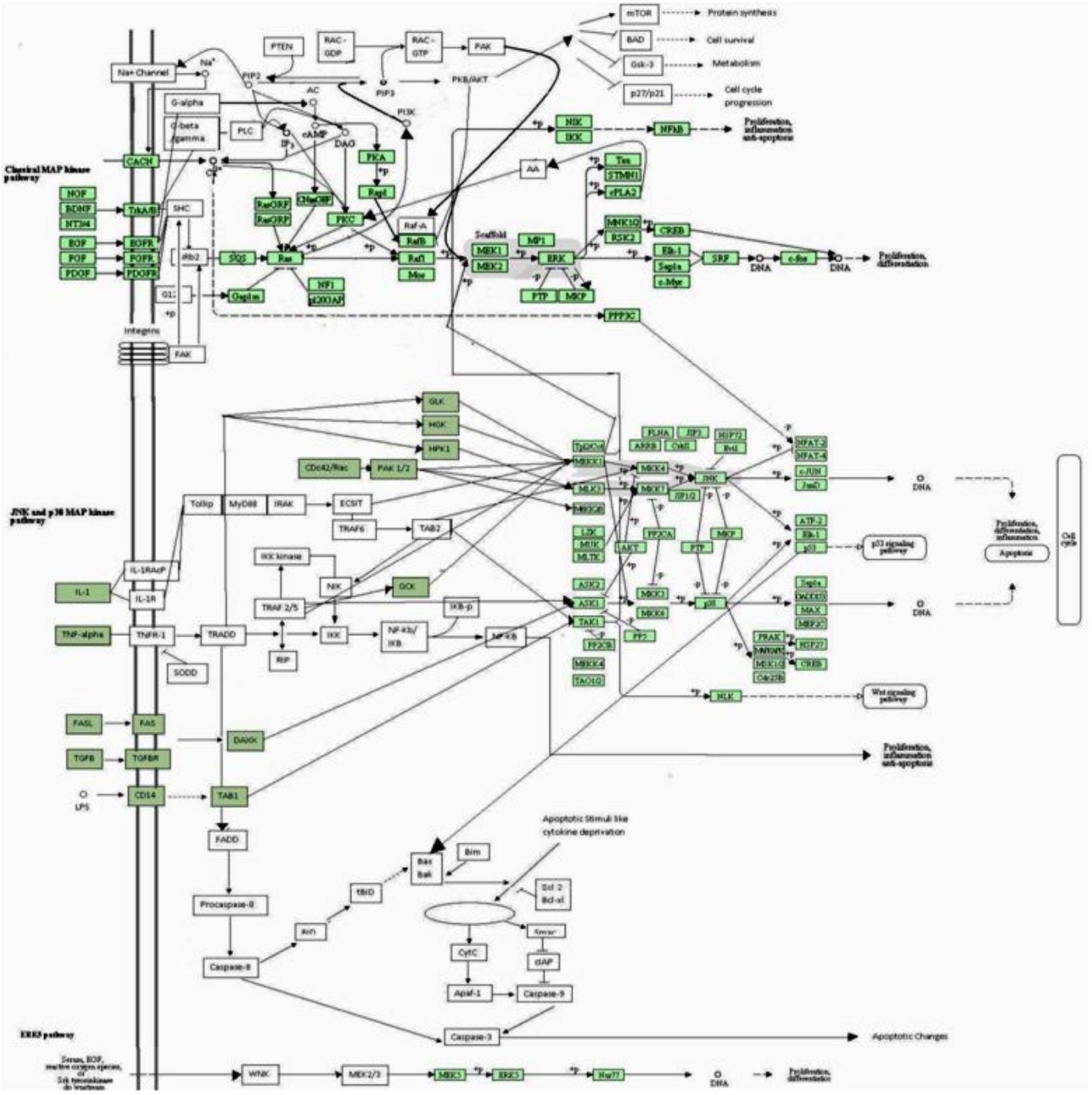

Figure 8: Curated MAPK pathway. The KEGG database pathway was taken as the basic model (green boxes) and more molecules and interactions were added (white boxes).

We obtained the following results after composing the network in CNA: 1) Basic Topological Properties of the complete network (see Table 5); 2) Interaction Matrix: Interaction of each species in each reaction shown by a color coded matrix, gives a holistic view of all interactions; 3) Dependency Matrix: Interdependence of each specie on every other specie shown by a color coded matrix.

Table 5: Basic network properties of MAPK pathway

Basic Topological Properties	
Species without any predecessors (Sources)	**37**
Species without any successors (Sinks)	**18**
Species connected to no reactions	**0**
No. of +ve feedback loops	**15**
No. of –ve feedback loops	**4**
No. of strongly connected components	**22**
Mutually Excluding pairs	**119**
Enzyme Subsets	**4**

These results give us a holistic view of the complete pathway but due to large number of species there is reduced readability and so we cannot do a detailed analysis. Hence we need to identify the most probable key species by extracting data from these results and then analyze them.

Identifying key species

We considered three parameters for identifying the key species: 1) Species interacting with more number of species; 2) Species influencing low number of species but crucial ones for cancer pathways; 3) Transcription factors leading to important cellular responses. We extracted this data by two methods: i) We divided all the species in three groups: Input Molecules, Intermediate molecules, and Output Molecules; and (ii) then we see the number of interactions in each group separately keeping in mind our desired parameters. After identification of key species in all the three groups we then quantified the interdependence of each of those species (see Figure 9). In the second method we quantified the number of transcription factors that lead to certain cellular responses and other crucial pathways.

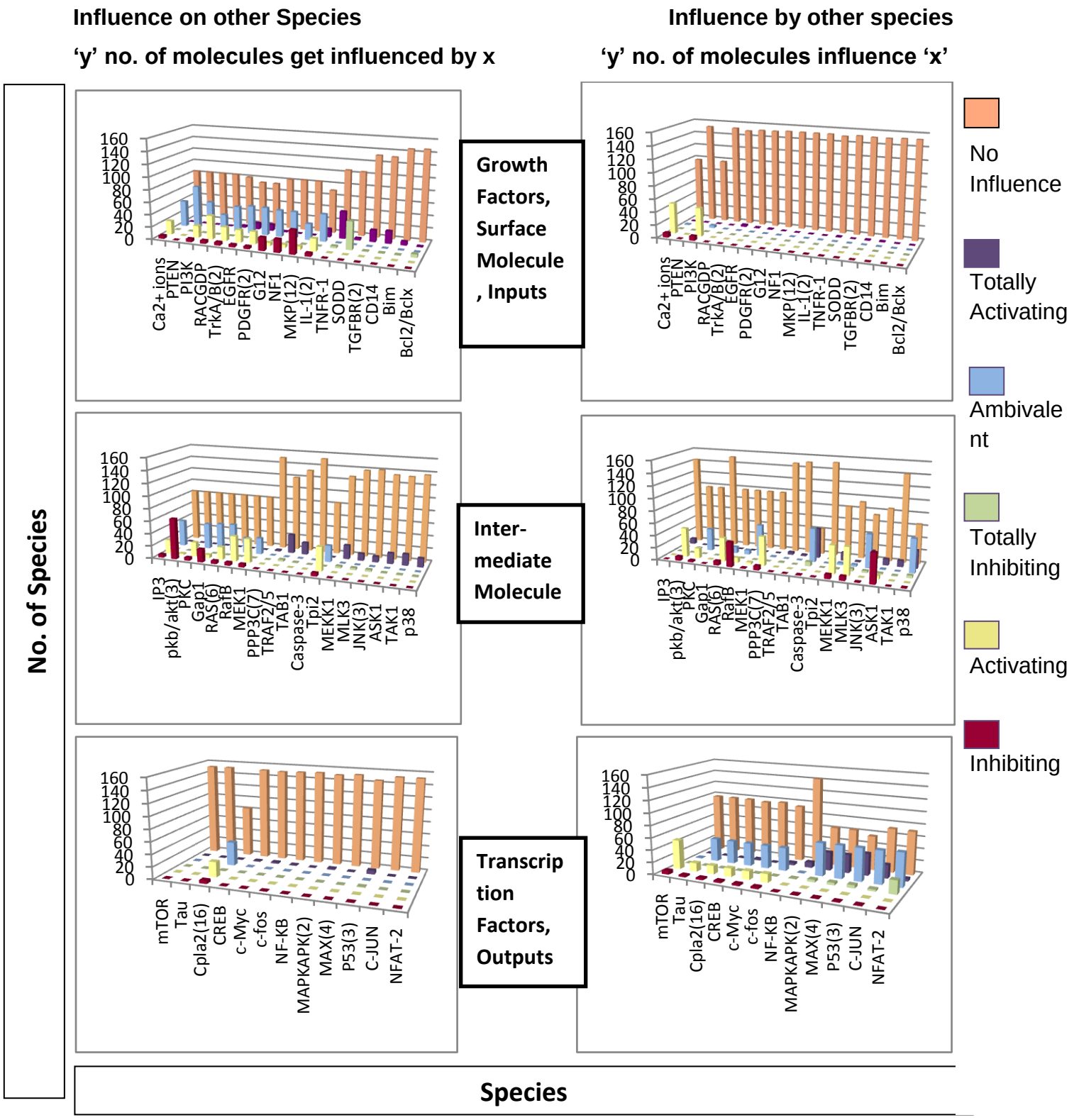

Figure 9: This is an attempt for obtaining quantitative results. Hence ennumerates the no. of molecules depending and influencing a particular specie.

Systematic perturbation study

After obtaining a basic idea of key species we perturb the normal states and thus do simulations. Hence we try all combinations, block (state = 0) and activate (state = 1) certain species to observe variation in results (cellular responses). Thus we try to identify alternative pathways and optimal intervention points. We observed some interesting results (See Figure 10).

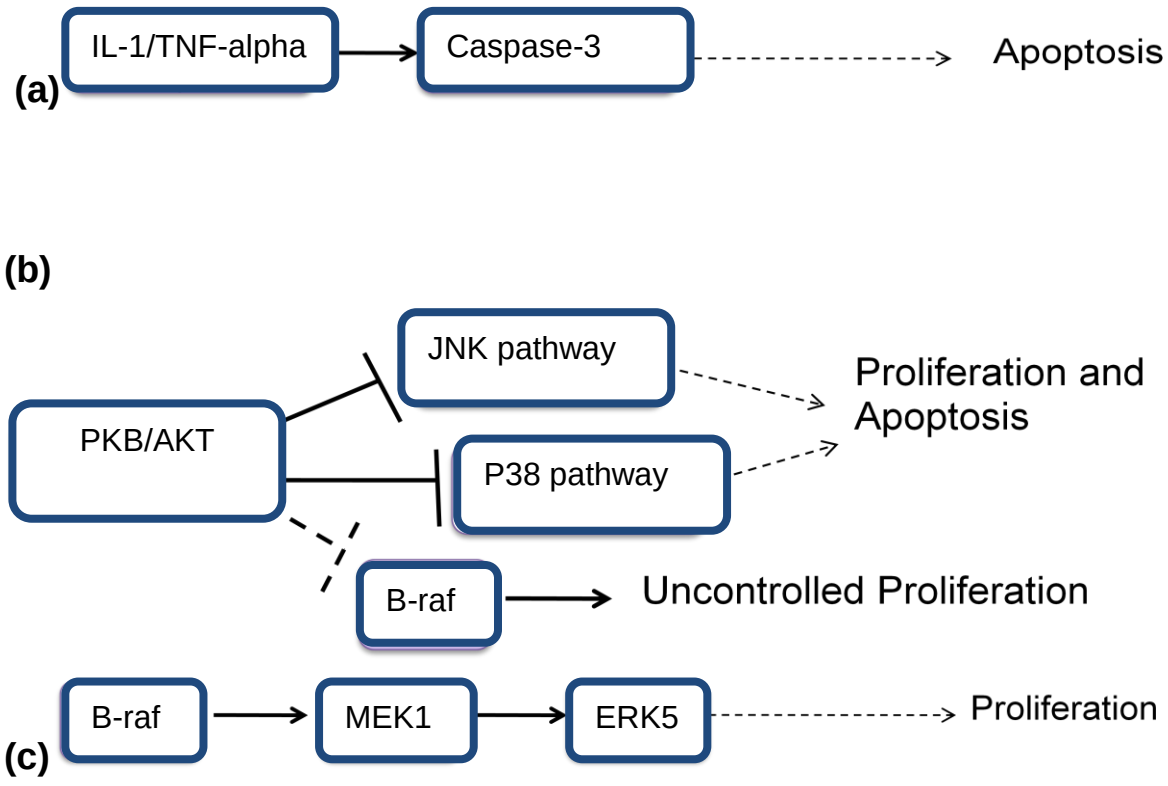

Figure 10: (a) IL-1: Interleukins (b) PKB: Protein Kinase B, JNK: c-jun Kinase pathway (c) MEK: Map Kinase Kinase, ERK: Extracellular regulated kinase (MAPK)

Inference

This study aimed at reconstructing the MAPK pathway to obtain an accurate and comprehensive pathway. This needs to be done before we further do any other analysis. After we modeled our reconstructed pathway we verified some simulations with published results and observed consistent results implying our pathway is correct. The software gives us only qualitative answers, such as network properties, feedbacks and so on. Hence extracting certain information we also tried to do a quantitative analysis, giving us a clear idea of the most probable key species.

From our complete study we observed that the MAPK pathway is closely connected and leads to crucial and drastic responses with just a small change. Hence by perturbation study we identified alternate paths to normal ones already recognized. Simultaneously we tried to identify optimal intervention points which can be further analyzed as probable drug targets.

After a systematic perturbation study we observed that Interleukin-1 has an independent pathway to apoptosis (See Figure 10(a)). Hence would be helpful in killing the cancerous cells. We also observed that PKB/AKT inhibits JNK & p38 pathways which are pathways to a balanced cell cycle. Hence if we inhibit PKB (one molecule already identified – NKX-3 [23]) it will allow JNK & p38 to be activated and hence normal cell cycle will be observed. PKB/AKT also negatively regulates B-raf which is a molecule that leads to uncontrolled proliferation (See Figure 10(b)). Since this just negatively regulates and does not inhibit the process of uncontrolled proliferation it is not a very good target rather it would be better if we can find a direct target to control proliferation, i.e., for example inhibit b-raf (See Figure 10(c)).

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