Stability Analysis of p53 Core Gene Regulatory Network Using the Frame work of Hybrid Automata

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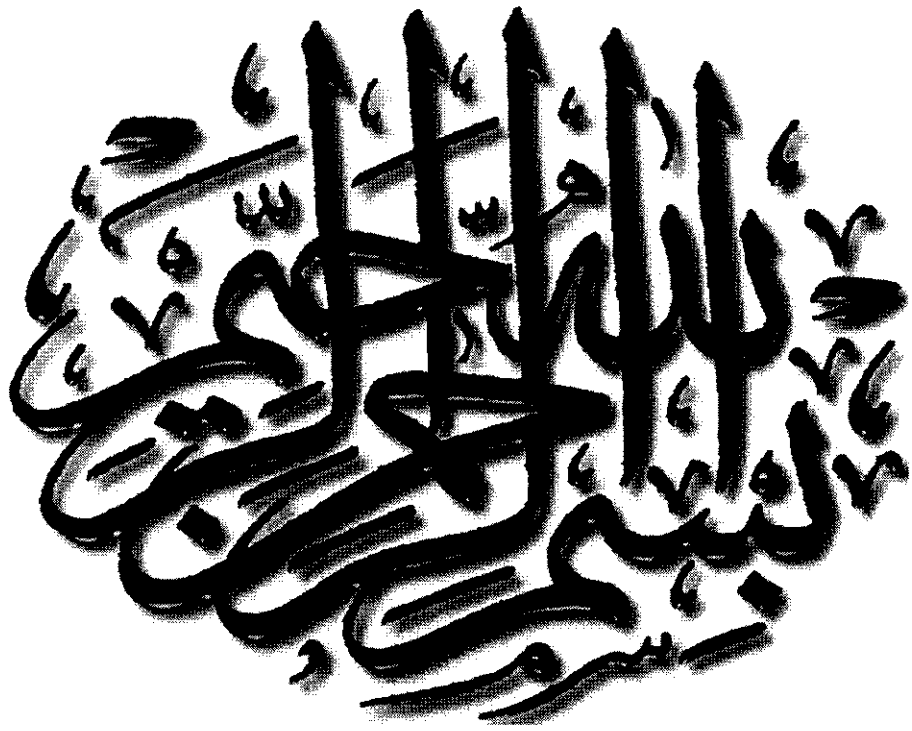

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FINAL APROVAL

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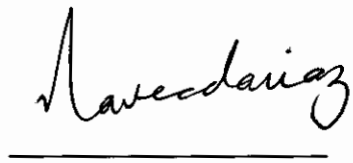

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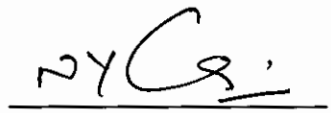

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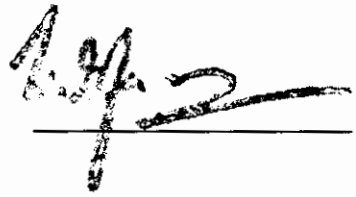

A thesis submitted to Department of Environmental Sciences, International Islamic University, Islamabad as a partial fulfillment of requirement for the award of the degree of MS Bioinformatics

This thesis is dedicated to my Mom, Dad and my Brothers for their endless love, encouragement, support, counsel & everything they did for me

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Dated: 03-07-2012

Syeda Uzma Ali

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LIST OF ABBREVIATIONS

Acronym	Abbreviation
Biological Regulatory Network	BRN
Gene Regulatory Network	GRN
Deoxyribonucleic acid	DNA
Ribonucleic acid	RNA
Liner Hybrid Automata	LHA
Mouse Double Minute two	Mdm2
Double Strand Breaks	DBS
Ataxia Telangiectasia Mutated	ATM
Alternative Reading Frame	ARF
Ataxia telangiectasia related	ATR
Ultraviolet	UV
Period	π
Real Time Modeling	RTM
Generalized logical networks	GLNs

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ABSTRACT

Cancer is anticipated as the preceding cause of death all around the world. The tumor suppressor gene Tp53 plays critical role in the suppression of tumors. The p53 is the essential protein in the response of DNA damage and is the element of a complex gene regulatory network. Regulatory networks act as skeleton offering a qualitative structure, on which kinetic logics rules applied to carry out quantitative modeling and simulation. The P53 feedback loop responds to stress that can interrupt cell division and DNA replication. The p53 is activated by posttranslational modification as reaction of stress signal, results in cell cycle arrest or cellular apoptosis. This process is under the control mechanism of p53-Mdm2 feedback loop. The modeling of the p53 network using discrete framework and through discrete automata helped in understanding fully the regulatory mechanism involved in the working of p53-Mdm2 feedback loop in cancer studies.

The qualitative modeling formalism of Rene' Thomas is a well know procedure to observe the dynamic behaviors of biological regulatory networks. We first apply this formalism to observe all steady state behaviors of the biological network containing p53-Mdm2 feedback loop and then apply a hybrid modeling formalism to accurately analyze the invariance kernel, region of cyclic trajectories, to compute the conditions in the form of time delay constraints to have a better insight of the behavior of the network.

In this study, a regulatory network has been used to construct a discrete model. The regulatory network involves p53 and Mdm2 (inhibitor of p53), Moreover this GRN is use it to study the effect of stresses on p53. In response to stress the multi-valued model demonstrate a wide variety of detailed output behaviors of steady states with

high levels of p53 and its inhibitor Mdm2 as well as cycle (oscillation) with low or high level of p53 peaks. Oscillations are not observed in Boolean model suggesting that enhanced activity of ATM is necessary in order to maintain homeostasis to respond the stress.

Further our results suggest that in the invariance kernel, where p53 plays a vital role against the stress, that the production and degradation activity of p53 is faster than the production and degradation activity of Mdm2. Our results suggest that trajectories leading to stable steady state, results in the over expression of p53.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

The body system of living organism consists of trillions of cells, which grows and divides continuously, important for the growth and maintenance of the body. When these cells exposed to damaged or stressed they can cause a threat to the organism via DNA damage (Macleod, 2000). Depending on the threatening situation and severity of the stress cells will either fail to complete their normal function but still use resources, or in the most severe condition the cells become cancerous. There are systems to defend this threat by stopping the cell division and repairing the DNA. The cell cycle has incorporated controls system for deciding about the step of division. Such type of control mechanism is called the homeostatic mechanism. This type of mechanism has signals to decide whether to move in or exit from the cell cycle. The GO and STOP signals of this machinery are decided by proto-oncogenes and tumor suppressor genes respectively. STOP signals are encoded by tumor suppressor genes, enlighten a cell to stop dividing and run off the cell cycle while GO signals are encoded by the proto-oncogenes, that tells the cell to move in the cycle and keep on to dividing. Deregulation of these STOP or GO signals by any type of stress or damage can lead the cells to escape from the tight controls that maintain homeostasis. Cells that accumulate damaged DNA lose their capability to respond to or make STOP signals resulting in cancer development (Nikolova *et al.*, 2000).

Cancer is the most arrogant, highly destructive and proliferative tissue invasive and apparently immortal population of body's own cell (Harding, 2007). It has different manifestation in different tissue, which is of fundamental importance to patients and their clinicians (Hallinan *et al.*, 2004).There are at least 200 different types of cancer,

as many as there are cell types in the human body (Parkin *et al.*, 2005). The cancer is major health problem worldwide with around 10 million fresh cases and 6 million deaths per year (Ferlay *et al.*, 2010).

Ever since cancer is diagnosed, laborious research has been done for its cure and treatment. But still we are not safe from its adverse effect. In order to understand cellular function and devise new therapies, it is of worth importance to understand the mechanism of activity of the genes involved in the network because the expression and activity of genes are not independent or isolated from each other (Shmulevich *et al.*, 2001). When cellular DNA is damaged it activates signal transduction pathways as well as repair mechanisms that elicit cell cycle or apoptosis. Some of these reactions are mediated by activation and stabilization of the p53 (Agarwal *et al.*, 1998; Levine *et al.*, 1997; Gottlieb *et al.*, 1996). P53 is a tumor suppressor gene and is one of the most important genes in the study of cancer genetics. In more than 50% of human cancers it is mutated and its pathway is inactivated in most of the remaining cancers. As transcriptional activator it controls the expression of a range of genes significant in the regulation of cell cycle and apoptosis by eliminating cancer-prone cells from the replicative pool (Goodsell, 1999). P53 inhibitor, Mdm2 (Mouse double minute two) is also of great importance, its amplification or over expression results to inactivation of p53. Mdm2 or sometimes known as Hdm2 in humans is an ubiquitin ligase known as the key negative regulator of p53 (Wahl and Carr, 2001).

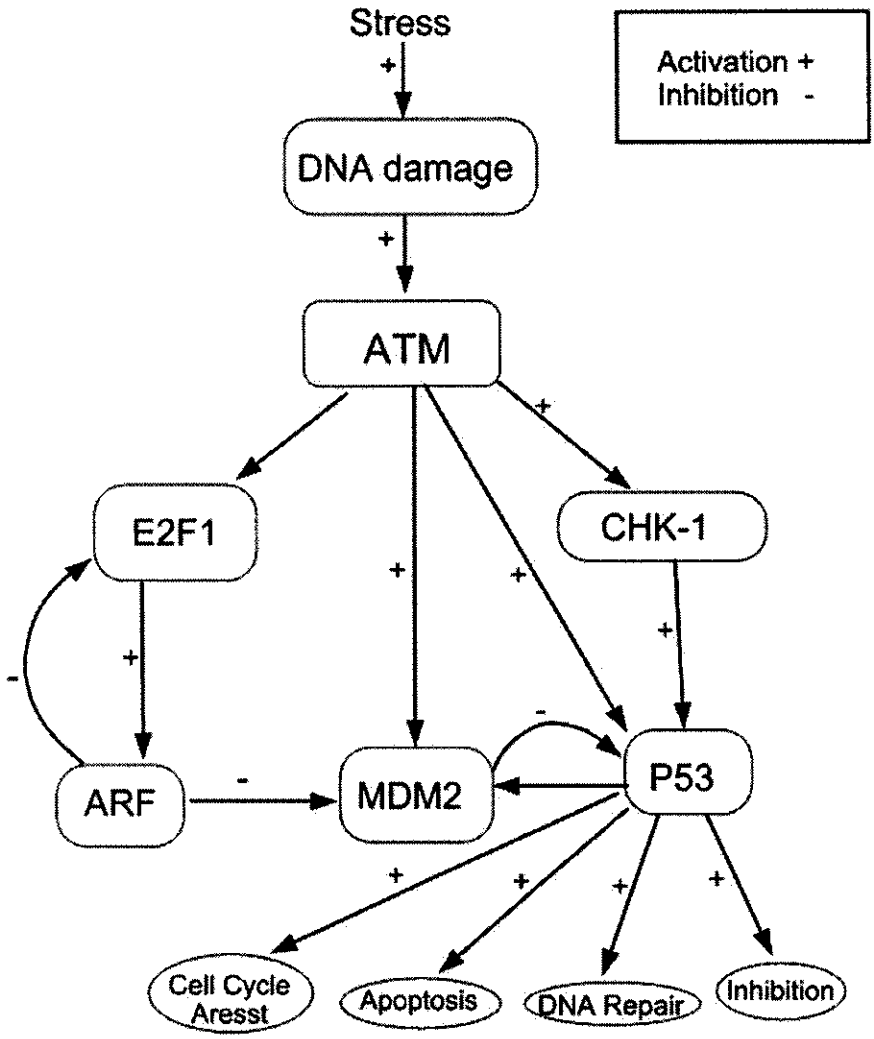


Figure 1.1: A diagram summarizing the key elements and interaction for the activation of p53

1.1. Key Elements of A P53 Gene Regulatory Network (GRN)

1.1.1 STRESS

The p53 pathway is activated in response to a variety of extrinsic or intrinsic stress signals. These comprise damage to DNA, shortening of telomere, low pool sizes of nucleoside triphosphate, hypoxia, damage to spindle, heat and cold shock, production of nitric oxide and inflammation, as well as mutations caused by activation of oncogenes (Levine, 1997; Vogelstein *et al.*, 2000; Jin and Levine, 2001). All above mentioned stresses would increase the mutation rates in cells by decrease the fidelity of progression in cell cycle and replication of DNA (Levine *et al.*, 2006). The signals that can harm the integrity of the DNA structure are Gamma or UV irradiation, alkylation of bases, depurination of DNA or reaction with oxidative free radicals. All these signals can alter the DNA by different means, and hence result in a different mechanism of detection and repair (Gudkov and Komarova, 2003; Oren, 2003). Damage to the DNA would be lethal if it is left unrepaired or it is mis-repaired. Mis-repairing of injured DNA can result in to the production of proteins with mutations, which can contribute to cells becoming cancerous. Therefore it is of very important for the cell to take action in the response of DNA damage. There are different DNA damage response pathways depending on the type of damaging agent (Harris and Levine, 2005). Recent studies have confirmed the subsistence of three independent and different pathways, through which p53 network can be switched on (Vogelstein *et al.*, 2000).

1. ATM (ataxia telangiectasia mutated) is the crucial protein to signals double stranded breaks in DNA (DSB in DNA) caused by gamma-radiation and ionizing radiations.

Both ATM and the CHK-2 kinase are activated in response to DSB in DNA and further phosphorylate the p53 protein. It is said that a single DBS in DNA may be enough to prompt a rise in levels of p53 (Vogelstein *et al.*, 2000).

2. The second pathway is activated in response to aberrant growth signals. It triggered by the over expression of some oncogenes e.g Myc and RAS (Vogelstein *et al.*, 2000). Oncogenes are supposed to stimulate cell growth and so over-expression of these oncogenes may result in making the cell cancerous. The over expressed oncogenes in humans is sensed by the protein called p14 ARF (Sherr and Weber, 2000).
3. ATR (ataxia telangiectasia related) is another DNA damage surveillance protein that is related to ATM (Abraham *et al.*, 2001). It is activated along with CHK-2 and casein kinase when DNA damage is caused by range of drugs used in chemotherapy, UV light and by inhibitors of protein-kinase (Harris and Levine, 2005). Early embryonic lethality or cellular in viability is reported in mouse, caused by the disruption of the gene for ATR (Brown and Baltimore, 2000).

The ultimate goal of all the mentioned pathways is to slow down the degradation of p53 and to stabilize it relatively high concentration by increasing its half life. Once the concentration of p53 is increased in cell, it binds to specific DNA sequences and activates the expression (transcription) of nearby genes. These expressed genes further play their role in arresting the cell cycle, DNA repair, apoptosis and inhibition of angiogenesis and metastasis (Vogelstein *et al.*, 2000).

1.1.2 Core of the Network

1.1.2.1. P53

The p53 is a transcription factor encoded by the TP53 gene in human through the process of translation (Matlashewski *et al.*, 1984; Kern *et al.*, 1991). P53 is important as it manage the cell cycle in multicellular organism and thus functions as a tumor suppressor and help in preventing cancer. Different terms have been used for p53 such as "guardian of the genome", "guardian angel gene" and the "master watchman" because of its role in preventing mutation in genome. The p53 gene was first identified as an Oncogene, or tumor promoter, and is found in mutated form in over 50 % of all cancers (Culotta *et al.* 1993).

Initially under normal conditions, the p53 protein is present in low levels as it is unstable. However, when the cell has to deals with stress, such as damaged cause by ultraviolet light , ionizing radiation or activation of oncogene, certain chemical groups are added to p53 which results in increases concentration and stabilization of p53 (Bode and Dong, 2004). P53 gene continuously monitors the integrity of the DNA molecule, and during the conditions of defects, steps are taken by it to either repair the errors or to kill the cell. If the repairs are possible then possibility of mutations and progression towards cancer would be reduced. In case if repair is not possible, the p53 protein will move the cell to enter in a pathway which leads to apoptosis, Around 150 genes are targeted by p53 to prevent proliferation of damaged cells.

1.1.2.2. Mdm2 (Mouse double minute two)

Mouse double minute two (Mdm2) in mouse or sometimes known as Hdm2 in humans is an ubiquitin ligase which is the transcription factor or negative regulator of tumor suppressor p53 (Wahl and Carr, 2001).The murine *Mdm2* gene contains at least 12 exons and its size is about 25 kilobases (Montes *et al.*, 1996). In humans the largest Mdm2 protein consists of 491 amino acids and has numerous conserved domains between different species (Marechal *et al.*, 1997). The first conserved region was identified in the amino-terminus of Mdm2 and its function is to intermingle with p53 and hinder its transcriptional activity (Olson *et al.*, 1993).

In several studies the Mdm2 gene is reported as an oncogene because of its behavior in human tumors. Over expression of Mdm2 is reported in a wide variety of human tumors. It might be due to one of these three different mechanisms: increased transcription, amplification of genes or enhanced translation (Landers *et al.*, 1997). Following are the types of tumors caused by the over expression of Mdm2: soft tissue sarcomas, glioblastomas and astrocytomas (Reifenberger *et al.*, 1996) osteosarcomas, (Lonardo *et al.*, 1997); oral squamous cell carcinoma, , acute myeloid leukaemias, breast carcinomas, acute lymphoblastic leukaemias and malignant melanomas (Poremba *et al.*, 1995).

1.1.3. Main Regulators of the Core

1.1.3.1. ATM (Ataxia Telangiectasia Mutated)

Several molecular studies have exposed the importance of ATM, it is thought to be a crucial component of intricate network the function of which is to senses the damage as well as other cellular stresses and then transmits these sensed signals to proper

effectors (Zhou *et al.*, 2000; Abraham *et al.*, 2001). ATM is the gene defective in ataxia telangiectasia, leads to human cancer predisposition and neurodegenerative syndrome (Shiloh, 2001 and Abraham, 2001). In an unstressed cell ATM kinase resides in the form of inactive dimer or in the multimer of higher order (Bakkenist and Kastan, 2003). When stress occurred ATM is recruited to the DNA damage site and become functionally active (Lavin *et al.*, 2005 and Bakkenist and Kastan, 2003). Functionally active ATM transmits the damage signal to the proteins in the network and phosphorylates the Mdm2 and p53 to disrupt the negative feedback loop (Norbury and Zhivotovsky, 2004).

1.1.3.2. E2F1

Protein E2F1 is a transcription factor, encoded by the gene *E2F1* in humans. The protein E2F1 belongs to the family of E2F transcription factors and is believed to be the first cloned member of this family. The member of this family plays a crucial role in regulating cell cycle and in action of various tumor suppressor proteins (Neuman *et al.*, 1996). Prospective E2F recognition sites [5'-TTTSSCGS] (where S = C/G) are found in the regulatory regions of various genes, responsible for encoding proteins which are required in replication of DNA and growth of cell (Slansky and Farnham, 1996).

1.1.3.3. ARF (Alternative Reading Frame)

Alternative reading frame (ARF) (known asp19ARF in mice and p14ARF in humans) is a tumor suppressor protein present at the *INK4a* locus. This locus is most frequently deleted chromosomal region in human tumors (Quell *et al.*, 1995). E2F1 has ability to inhibit Mdm2 so it serves as a key modifier in various p53 dependent signaling

pathways (khan *et al.*, 2000). Both in vivo and in vitro studies have helped in the identification of P14ARF as a potent tumor suppressor (Sherr, 2001). ARF is normally expressed at low levels but it is rapidly up-regulated by oncogenes during the replicative senescence process (Bates *et al.*, 1998; Carnero *et al.*, 2000).

1.2. Down Stream Events of the P53

Once the p53 protein is get stabilized and activated it starts accumulating in the nucleus and binds to particular DNA sequences in order to promote or suppresses the transcription of neighboring genes (Bode and Dong, 2004). It is said that p53 is a tetramer, potential to bind with specific sequences and hence result in transactivation of group of genes. Different studies showed that different promoters sense active p53 differently, resulting in differential DNA binding and transactivation. It also behaves as transcription factor, inhibiting the expression of some genes (Agarwal *et al.*, 1998). Several genes and group of genes that are directly regulated by p53 gene have been divided into four major categories.

1.2.1. Cell cycle Arrest

Cell cycle arrest or cell cycle inhibition in cell division is thought to be the fore most and effective response after the expression of p53. The p53 protein directly controls the expression of p21 that inhibits cyclin E-cdk2 (Vogelstein, 2000).

This cyclin-dependent kinase derepresses the activity of E2F1 by acting upon the Rb protein. E2F1 is the protein involved in the transcription of those genes which assist the cell in progressing through G1 to S phase in the cell division. 14-3-3 sigma and CDC2 are essential proteins that take part in arresting G2- M phase (Iliakis *et al.*, 2003).

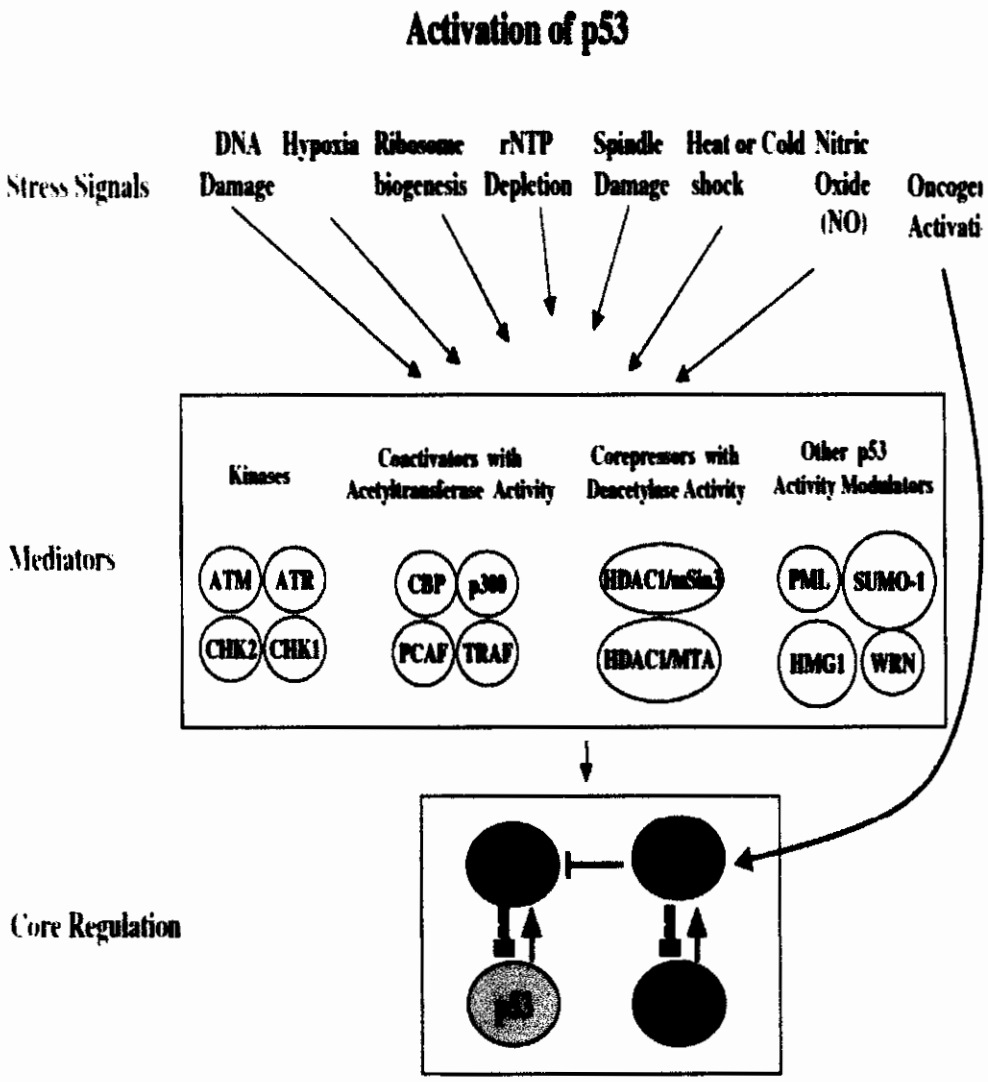


Figure 1.2: Varity of intrinsic and extrinsic stress signals that can activate p53

(Harris and Levine, 2005)

1.2.2. Apoptosis

P53 stimulates the expression of large number of genes involved in triggering programmed cell death (Agarwal *et al.*, 1998). These include proteins of the Bcl2 family, proteins involved in the induction of reactive oxygen species, proteins of death domain ,proteins of plasma membrane, PMP-22 (PERP), APAF1 and IGFBP3 (Wahl and Carr, 2001). An important member of the Bcl2 family is Bax, first apoptotic factor recognized as a target for transactivation of p53 (Hickman *et al.*, 2002). P53 can directly stimulate mitochondria that could result in death by producing excessive highly toxic reactive oxygen species (Vogelstein, 2000).

1.2.3. DNA repair

The p53 protein is of vital importance in maintaining genetic stability. Although its mechanism of activity is not clear yet, but it might entail the induction of genes that control 'nucleotide-excision', DNA repairs, segregation of chromosome and recombination in chromosomes (Vogelstein, 2000). The vital genes involve in mentioned processes are DDB2, p53R2 (p48), GADD45 respectively (Smith *et al.*, 1994; Tanaka *et al.*, 2000).

1.2.4. Inhibition of Angiogenesis and Metastasis

Several lines of evidence have suggested that triggering the expression of genes which are involved in preventing the formation of new blood vessels is normal function of p53 protein (Waldhoff *et al.*, 2000). The important genes involved in inhibition of blood vessels formation are PAI, BAI-1 and Maspin (Harris and Levine, 2005).

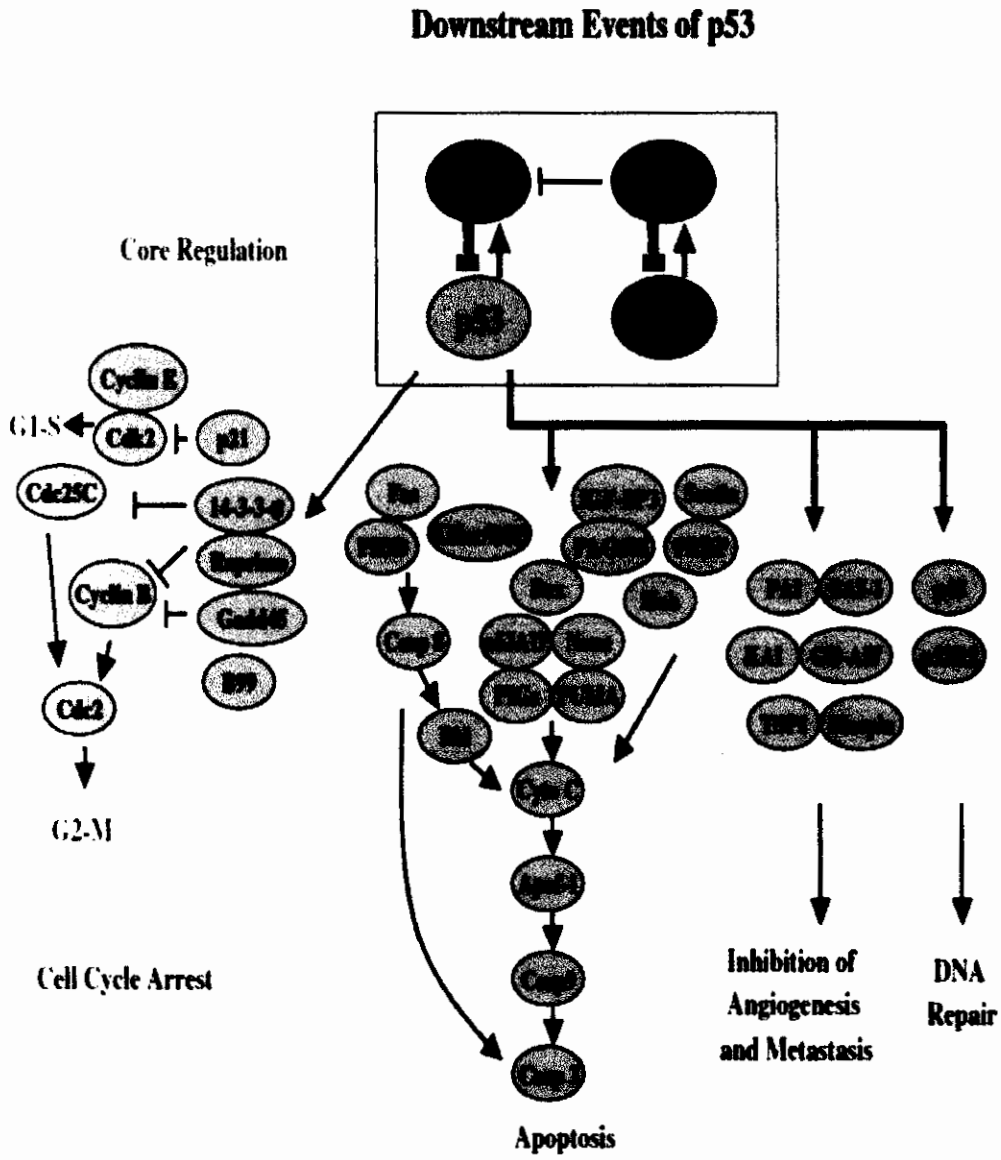


Figure 1.3: Downstream events of p53

(Harris and Levine, 2005)

1.3. Biological Regulatory Network

The organisms are subjected to evolution and over the time this evolution results in phylogenetic relationship among them, these relationship between the elements are of great interest. These relations can be described as biological networks. BRNs are graphs which represent interactions between biological entities, genes and regulatory products (proteins) as vertices and their interactions are represented with edges. These interactions are further bounded and signed (Ahmed *et al.*, 2009). At the molecular level biological networks are gene regulation network, signal transduction networks, protein interaction networks and metabolites network as shown in figure 1.4.

The biological networks combine compound interactions in the cell and give an overall or global view of within cell activities. The relationship between molecules can be well interpreted (Junker and Schreiber, 2008). Biologists embody biological system in terms of graphs and BRNs represent interaction between genes or their products. In living organism every process has two types of important network called gene regulatory pathways and signaling pathways. Gene regulatory networks (GRNs) portray connection between genes that is based on how the expression level of one gene affect expression levels of others. Genes do not interact directly with other in its place, gene stimulation occur through proteins which are also the product of genes, it can also be affected by metabolites directly (Helms, 2008). GRNs are graphs which represent genes and regulatory products as vertices of graph and their interactions are represented with edges. These interactions are further modified by making them signed and directed. The plus '+' sign show activation and minus '-'sign inhibition (Karlebach and Shamir, 2008; Ahmad *et al.*, 2006).

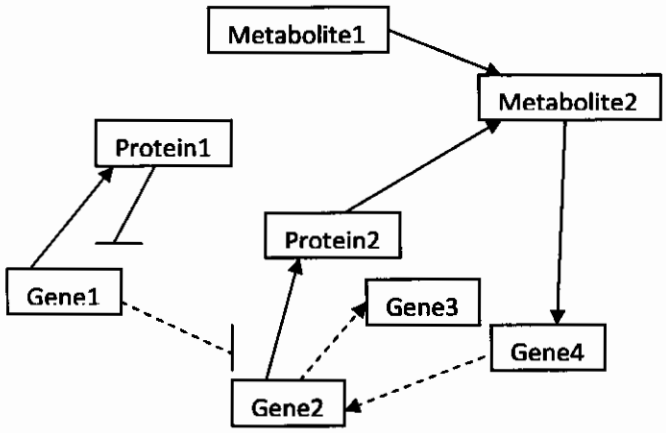


Figure 1.4: Model of Biological Regulatory Network (BRN)

1.3.1. Importance of Biological Regulatory Network

Analysis of Biological networks is of vital importance in order to understand their mechanism of action (Ay *et al.*, 2009).Gene regulatory network act as a skeleton and the development plane of body is maintained by huge gene regulatory network (Eric *et al.*, 2002). A regulatory network provides a qualitative framework on which quantitative data can be applied (Junker and Schreiber, 2008). To predict the dynamical behavior of regulatory networks we use modeling, modeling of these regulatory networks further help in understanding the functionality of their components, which can then be used for prediction of a set of nonobvious conclusions that can be experimentally proved after word (Cara *et al.*, 2007).

1.3.2. Dynamics of Biological Regulatory network

Jacob and Mond were the first who have started simulation and analysis of gene regulatory networks (GRN) (Jacob and Mond, 1961). The idea of interpreting gene interactions as logical systems can be used for further developments using Boolean networks and their generalization to discrete networks (Ahmad et al., 2007; Thomas, 1973). Theoretical models of GRN can be used when complex behaviors are generated by the interaction of genes involved in various feedback loops. In case of these complex behaviors, understanding the observed phenotype is impossible without the aid of computer, use of computer to perform modeling and simulation can help the biologist in exploring possible behaviors of observed phenotype (Bernot *et al.*, 2004). For the last three decades GRNs are studied by pioneering modelers who have provided evidence in favor of role of dynamics in the systems. Models of network having ten genes have been formed. Either through differential equations (Gonze *et*

al., 2002; Tyson *et al.,* 1999) or discrete frame work of Boolean networks (Kauffman, 1993) or discrete automata (Thomas, 1999). They have helped a lot in better understanding of some of the major regulatory mechanisms involved in cell for example producing a broad view of dynamical phenomena responsible for epigenetic switches, (Richard *et al.,* 2005; De Jong, 2002).

The recognition of stable and unstable steady state, limit cycles, classification of multi stable behavior and identification of oscillatory behaviors are the properties of qualitative (Tyson *et al.,* 2003) . It also include depiction of the function of the network in terms of signals i.e amplifiers, deviators, logic gates, and valuation of environmental changes or genetic mutations (Tyson *et al.,* 2003, Wolf and Arkin, 2003)., The default option for modeling regulatory process is to use and define a differential equation. In differential models of GRNs, the activity of gene is represented by a concentration of the associated RNA or proteins x_i, and the evolutions (function) of all concentrations follow a differential equation system dx/ dt = f(x). Biologists can take advantage from these observations leading to forming highly non-linear models with some robust threshold effects. Differential equation systems have major drawbacks. They cannot be solved analytically as they are mostly nonlinear. In addition, often the experimental data is of qualitative nature and is not suitable for evaluation of quantitative parameters for the differential model (Siebert and Bockmayr, 2006). The derivation of the dynamics from the interaction graph is not trivial as inference of lot of parameters required, this lead to modification and a tiny modification of a parameter can lead to a strong change in the dynamics. Each equation requires knowledge of one or several parameters and it is difficult to instantiate models of large networks (Ahmad *et al.,* 2006; De Jong, 2002). To

overcome the limitations kinetic models or discrete models are used. Piecewise linear differential equation is the part of simplified continuous framework. It is a special rate equation in which response of gene to regulatory stimuli is approximated by the use of step function (De Jong, 2002). Linearity of equation is used to solve some qualitative properties such as steady states. It can be analyzed qualitatively by discretization and recasting them into the framework of qualitative differential equation in which variable and their derivatives have discrete values. In kinetic models, modeler can discretize the concentration and can highlight the effects of threshold (i.e. use of delays). It includes Boolean networks and generalized logical networks (GLNs). The Boolean network is representation of system consisting of n variable and nodes; they take two values 0 and 1. 0 for unexpressed and 1 for expressed state, and set of logical rules together describe evolution of a system from a current state at time t to the next state at time (t $+\Delta$) (Ahmad *et al.,* 2006). GLN is the generalization of Boolean network in which variables can have more than two values and asynchronous transitions (Thomas *et al.,* 1995). The generalized logical analysis built by Thomas and co-workers (1995) to illustrate biological network dig out the essential qualitative features of the dynamics of such systems by logical parameters. Discrete models present a qualitative description of a system dynamics and focus on the structure of the system. Yildirin and Mackey developed the theoretical model for time delay (Yildirim and Mackey, 2003). From a biological perspective, time delay in a gene regulation arises from delays caused by processes such as transcription, translation, and transport processes. Accounting this attribute is necessary to accurately capture the dynamics of the system (Altman *et al.,* 2004). Biological regulatory mechanisms include an important concept of time delay, particularly in case of transcription

factors. In bio-molecular level transcription to mRNA and protein translation involves a time delay (Huang *et al.,* 2010). In kinetic logic, the on and off delays for a given gene will generally be unequal, and the delays of different gene will also be different. This shows that biological systems have asynchronous behavior, i.e., all the genes forming a system will not be transcribing at the same time.

1.3.3. Advantage of Dynamic systems

The dynamical system used for the construction of GRNs can assist the researchers in finding the association between biological systems and general dynamical theory. A wide Varity of influential analytical and theoretical tools are available to explore the dynamical system (Thomas, 1999; Tyson *et al.*, 2003). This approach can be used to categorize the key components, interactions and variables in order to determine the behavior of the system thus providing better insight to all possible targets .It simulates experiments before performing them, thus saving a lot of time and money which is used in performing hit and trial experiments in wet labs. It avoids doing experiments on animals. Models can be refined by comparing experimental measurements with simulated dynamics. It provides insight on qualitative attributes of the system (Bernot *et al.,* 2007).

1.3.4. Linear Hybrid Automata

A Linear Hybrid Automata is a subclass of Hybrid Automata, having full automation. A Hybrid Automata X is linear when all the constant and initial conditions are convex linear predicates over A, all the flow conditions are convex over A and all the skip conditions are convex over A U A' , where A' = $\{a_1', \ldots, a_n'\}$. In case a predicate is an inequality having rational coefficients, over variables in a set Q is linear. If a predicate

is a finite set of linear predicates then it is a convex linear predicate. This definition shows that a linear differential equation cannot be inevitably translated into a linear flow condition as there are distinctive concepts of differential equations and hybrid automata (Uller and Stauner, 1996).

In HYTECH, model checking algorithm is implemented to analyze a property such that whether it is violated or not in any state. It sorts out the set of states of Linear Hybrid Automaton by repeatedly applying the time and transition steps, which are approachable from the set of initial states. It can also execute backward accessibility analysis by using time and transition steps, of such a path from where the final path is reachable. The algorithm applied in HYTECH may not necessarily be completed as it is semi-decidable process (Uller and Stauner, 1996). HYTECH is used to analyze the models with pre-defined parameters.

Qualitative approach can be applied to the interactions that require parameterization (Thakar *et al.*, 2010). For systems having both qualitative and quantitative behavior, then LHA is a very popular modeling formalism as it has the features of both the continuous as well as discrete system (Grosu *et al.*, 2007). It is derived from finite-state automata. The discrete states show numerous approaches of continuous dynamics of a system and transition correspond to swapping logic between these approaches. It is recently being used in modeling and analyzing biological systems such as GRNs, protein signaling pathways (PSPs), metabolic pathways etc. (Bemporad *et al.*, 2007).

1.4. Proposed Biological Regulatory Network (BRN)

The biological systems are complex in nature. The major challenge in modeling BRNS is to deciding about the suitable level of detail to include. Including too much detail may leads to complicated model with reduced explanation, in which the essential nature of the process of interest might be obscured. On the other hand, including too little detail might be risky because it can omit significant processes and mechanisms, resulting in a model with imprecise representation of the real system (Geard and Willadsen 2009). The complexity in modeling p53 is due to the reason that p53 itself is very complex on many levels and so is its network of interactions (Braithwaite *et al.*, 2005; Kohn and Pommier, 2005). In different tissues and cell lines the p53 network behaves differently (Bouvard *et al.*, 2000; Fridman and Lowe, 2003) and its importance varies in different cells (Hickman *et al.*, 2002). Another complication is that both p53 and its negative inhibitor Mdm2 belong to a large family of proteins thus making the 53-mdm2 loop more complex (Michael and Oren, 2002). The p53 network is not independent or isolated in a single cell but is also affected by other cells through different growth and survival factors (Haupt *et al.*, 2003). The current study will be on the network that is activated in response of DSB in DNA.

The different types of intrinsic and extrinsic stress factors that can switched on the p53 pathway through ATM kinas include radiation and alkylation of oxidative free radicals and bases (Bode and Dong 2004; Gudkov and Komarova 2004; Appella and Anderson 2001). ATM is a member of a family of protein kinases. The members of this family have a phosphoinositide 3-kinase- domain at their carboxyl termini. The function of these enzymes is take part in managing genome stability, progression

through cell cycle, and activating certain genes in response of DNA damage (Kastan and Lim 2000; Shiloh, 2001). It is reported that deficiency of ATM in humans result in the genetic disorder ataxiatelangiectasia (A-T).This disorder is characterized by instability of genome, immunodeficiency and neurodegeneration, and, predisposition of cancer and sensitivity to IR (Khosravi *et al.*, 1999). the p53 protein is phosphorylates on serine 15 by ATM. The phosphorylation results in conformational changing in p53, making it more resistant against the inhibition of Mdm2. It is also reported that conformational shifting induced by ATM enhances the transcriptional activity of p53 (Maya and oren, 2001). *It* seems that ATM kinase not only induced modifications in p53 but also to its negative regulator Mdm2. Indeed Mdm2 can be phosphorylated on multiple sites (Buschmann *et al.* 2000). In order to repress transactivation activity of p53 and to target it to proteasome-mediated degradation, Mdm2 has to bind with its amino terminus. The destabilization process of p53 requires both the carboxyl regions and amino--terminal of Mdm2 for binding with it (Kubbutat *et al.*, 1999; Tao and Levine 1999). Observations suggest function of Mdm2 as an E3 ubiquitin-protein ligase in the process of p53 degradation. Similar to the phosphorylation of Ser15 on p53 (Siliciano *et al.*, 1997) .ATM also mediated the phosphorylation of Mdm2, which precedes the initiation of damage-induced p53 accumulatio. The speedy phosphorylation ATM targets leads to the immediate enhancement of ATM's kinase activity (Banin *et al.*, 1999; Canman *et al* 1998). Thus, ATM is the central player in the pathway and has impressive effect on activity and stability of p53 by performing parallel phosphorylation of p53 and Mdm2 (Khosravi *et al* 1999).

1.4.1 P53- Mdm2 feedback loop

The expression ofP53 is controlled by different other genes or proteins at two distinct levels. The first level is its stability, the proteins involve in p53's stability or to decrease its half life are Pirh2, COP1, Mdm2 (Leng *et al.*, 2003; Barboza*et al.*, 2008; Dornan *et al.*, 2004) and p53's activity is the second level of its regulation, the proteins which also function as transcription factor and regulates its activity are MdmX, Mdm2 (Shvarts *et al.*,1996). Mdm2 is directly involved in regulating both its mechanism of activation and stabilization. Mdm2 is reported an E3 ligase that binds to p53 and its transcriptional activity is inhibited through Mdm2 Mono-ubiquitination. While polyubiquitination of p53 by Mdm2 triggers its degradation (Li *et al.*, 2003). The significance of Mdm2 in regulating p53 is best understood by the study in which it is reported that Mdm2 deletion in mice has caused embryonic lethality, this lethality was rescued by deletion of p53, illustrating the fact that Mdm2 has major role in controlling the activity of p53 through ubiquitin-mediated proteolysis (Koji *et al.*, 2008). One of the important function ofMdm2 is to attaches ubiquitin molecules to the p53 protein and these molecules acts as a label for the protesome machinery in order to degrade the p53 protein (Vogelstein *et al.*, 2000). Mdm2 itself is a p53 target gene and so p53 and Mdm2 form a negative feedback loop. An increase in level of p53 will cause a rise in Mdm2 which in turn will inhibit p53 and reduce its amount. This feedback loop works to keep the p53 near to desire level (Harris and Levine 2005).

Objectives

Regulatory network associated with p53 pathway has been identified so for

1. Identified BRN will be further used in discrete modeling/ Hybrid modeling formalism application, based on the qualitative characterization of the biological regulatory pathways.
2. Building and analysis of Qualitative Models; it will require model checker tools for the qualitative analysis of the discrete/ Hybrid models. After model generation and parameter optimization, by performing in-silico experiments and adjusting similar biological observed characters, parameters will be identified.
3. The research will focus on behavior of the system, and for both normal pathways i.e. Oscillation and abnormal (diseased) condition i.e. dead lock state.
4. The second half of the study is based on the development of the Hybrid model of the system using regulation Delays (Production/ Degradation delays). In this part delays constraints and clocks will be introduced for the real time modeling by using Hytech software.

CHAPTER 2

MATERIALS AND METHODS

2.1. Dynamical Modeling

BRNs are graphs which represent genes and regulatory products (proteins) as vertices and their interactions are represented with edges. The interactions are directed and signed, positive (+) for activation and negative (-) for inhibition (Ahmad and Roux, 2009; Ahmad *et al.,* 2006; Dynlacht and Blais, 2005). Figure 2.1 (left) and 1(right) showing activation and inhibition in the form of increasing and decreasing sigmoid. The interactions in a BRN may lead to regulatory circuits. The circuits are of two types: positive or negative circuit. A circuit is said to be negative if it has odd number of negative interactions otherwise it is positive (Thomas and D'Ari, 1990). Occurrence of a positive circuit in a pathway represents epigenetics or multistationarity. Negative circuits symbolize homeostasis or stable fluctuation. Both are necessary and sufficient conditions in a qualitative model (Kaufman *et al.,* 2007; Thomas, 1981).

Logical system is a combination of a set of conjecture rules defined in systematic language. Here logic is associated with element of system by,

- A logical variable which shows level e.g., concentration.
- A logical function reflecting evolution e.g., evolution.

$$X = \theta(x, y, z, \ldots.)$$

Where θ = Logical function

$(x,y,z,\ldots.)$ = Variables

In biological system, it is most often used to deal gene expression. Dealing with gene gives gene on or off and their product present or absent.

X = 1 or 0 representing gene, 'On' or 'Off' respectively.

x = 1 or 0 representing gene-product, 'Present' or 'Absent' respectively.

Suppose a protein x is activated if and only if (iff) protein y is present and y is activated iff x is absent and y itself is present as drawn in Figure 2.1.

Mathematically,

$$x = y$$

$$y = \bar{x}y$$

Such expressions are represented by formalism,

The formalism of qualitative modeling defined by René Thomas (Thomas and D'Ari, 1990) is a well-known formalism to construct a discrete (qualitative) model of a BRN. It is given in the form of set of definitions and explained using an example as shown in Figure 2.2.

2.2. Dynamical Modeling of p53 Related BRN

After having extensive literature survey, p53 related biological network is constructed as shown in figure 1.1. The constructed model has all important genes which are activated or inhibited after DNA is damaged. As discussed earlier in chapter 1 that in unstressed cells the p53 network is normally 'off '. It is switched on only when cells are stressed or damaged. Such cells pose a threat to the organism as they are more likely to contain mutations and exhibit abnormal cell cycle control as compare to undamaged cell, thus having greater risk of becoming cancerous. The p53 protein inhibits the division of stressed cells by arresting the cell cycle. In order to protect the organism it can even cause programmed death (apoptosis) of cells contained damage DNA therefore providing critical brakes in tumors development (Vogelstein et al., 2000).

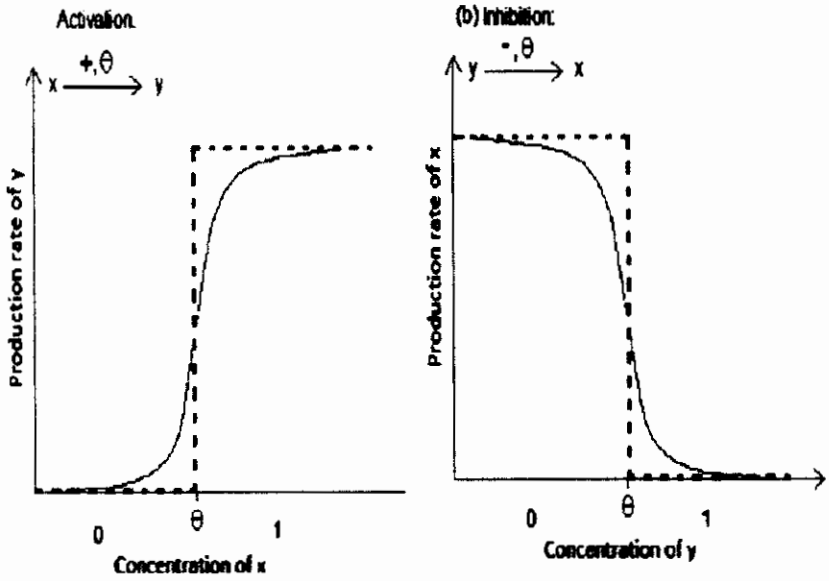


Figure 2.1: Activation curve (left) Inhibition curve (right).

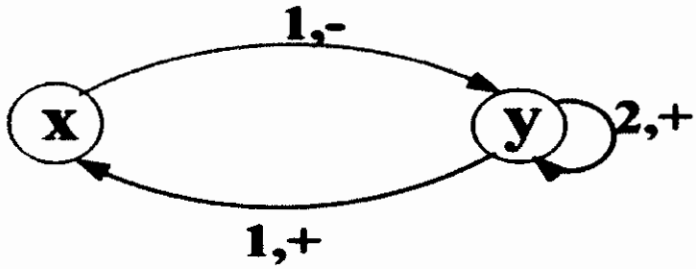


Figure 2.2: BRN showing the interactions for activation and inhibition x and y

x	y	w_x	w_y	$K_{x,wx}$	$K_{y,wy}$
0	0	{}	{x}	0	1
0	1	{y}	{x}	1	1
0	2	{y}	{x,y}	1	2
1	1	{y}	{}	1	0
1	0	{}	{}	0	0
1	2	{y}	{y}	1	2

Figure 2.3: State table of x and y BRN

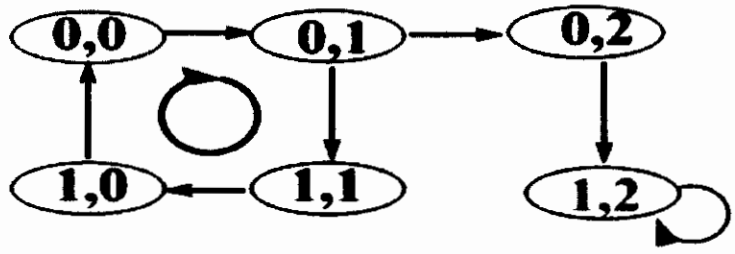


Figure 2.4: state graph of x and y BR

Different stress signals activate the p53 network in different way, depending on the damaging agent different detection and repair mechanism is activated. Several lines of studies have confirmed the activation of at least three different pathways in case of different stress signals (Harris and Levine, 20005). The pathway used in current study (figure 2.1) is activated in response to DNA damage, such as that caused by ionizing radiations or gamma radiations.

2.2.1. Abstraction of BRN

Abstraction of BRN (figure 1.1) is done by using the Transitive property of mathematics. If A activates B and B activates C then it can be assumed that A can directly activates C and the entity B can be removed if its concentration does not affect other proteins activation or inhibition in the BRN (respectively for inhibition).

Analyzing the BRN (figure 1.1) one can see that p53 is activated by ATM through two different pathways: one is direct activation of p53 by ATM and another through kinas chk1. ATM activates chk1 which arrest cell cycle and further helps in activating p53. So we will assume here the direct interaction and skip the other one. Similarly in the case of Mdm2 inhibition two different pathways emerged from ATM. First is direct phosphorylation of Mdm2 by p53 and the second is through E2F1-ARF negative feedback loop. The ultimate purpose of both the pathways is to inhibit the Mdm2. So again we will use the direct interaction and elude the other.

2.3. Dynamical Modeling Formalism

This section briefly explains the discrete approach for the analysis and modeling of BRNs. We have used formalism of Ren´e Thomas (Thomas, 1991) for BRN to derive

the qualitative state graph of a BRN. The state graph elucidates steady states which are of extensive importance for biologist.

2.3.1. Biological Regulatory Network

In a directed graph $G = (X, A)$, $G^-(v)$ and $G^+(v)$ represent the set of predecessors and successors of a node $v \in X$ respectively. A BRN is a graph $G = (X, A)$ where X represents the set of nodes (biological entities) and A is the set of edges representing interactions between biological entities. Each edge $a \rightarrow b$ is labeled as (s_{ab}, r_{ab}), where s_{ab} is a positive integer representing a threshold and $r_{ab} \in \{+, -\}$ shows the type of interactions ('+' for activation and '-' for inhibition). There is a limit lm_a for each node a which is equal to the outgoing degree of a, such that $\forall\ b \square\ G^+(a)$ each $s_{ab} \in \{1, \ldots, n_a\}$ where $n_a \leq lm_a$. Each entity a carries its abstract concentration in the set $Q_a = \{0, \ldots, n_a\}$.

To analyze the behavior of a BRN, it is necessary to know all the possible states and transitions between them.

2.3.2. States

A state s of a BRN is a tuple where $s \square \square$, such that

$S_0 = \Pi_{b \varepsilon X} Q_b$.

A vector is normally used to show a qualitative state $(v_b)\ \forall\ b \varepsilon X$, where v_b represents the concentration level of the product b.

2.3.3. Resources

A set of resources represents the activators of a variable at any instant. The set of resources k for a variable $a \varepsilon X$ at some level y is defined as

$K_{ya} = \{b \varepsilon G^{-}(a) \mid (y_b \geq s_{ba} \text{ and } r_{ba} = \text{'+'} \text{ or } (yb < s_{ba} \text{ and } r_{ba} = \text{'-'})\}$.

From the above definition, it can be inferred that the absence of an inhibitor is considered as an activator. The set of parameters assigned to a biological entity determine the dynamics of a BRN which is defined as:

$K(G) = \{K_{a, Rya} \in \{0, \ldots n_a\} \mid y_a \in Q_a \forall a \in X\}$.

$K_{a, Rya}$ gives the level towards which a evolves.

Let y and $K \in Z_{\geq 0}$, the asynchronous evolution operator $\uparrow$ is given as:

$$y \uparrow K = \begin{cases} y + 1 \text{ if } y < K \\ y - 1 \text{ if } y > K \\ y \text{ if } y = K \end{cases}$$

2.3.4. State graph

- If y_x is the level of an entity x in state $s \varepsilon S_0$, the state graph of a BRN with Transition relation $T \subseteq S_0 \times S_0$ such that
- $s \rightarrow s' \in T$ iff:
- There is a unique $a \in X$ such that $s_a \neq s'_a$ and
- $s'_a = s_a \uparrow K_{a, Rya}$
- *and*
- $s'_b = s_b \forall b \in X_{\setminus\{a\}}$.
- A State graph differs from its successor state by one component only, so if a state s has n elements to be evolved then it will have n successor states.

2.4. Logical parameters of the Biological Regulatory Networks

To observe the dynamical behavior of the BRN, **GENOTECH** tool was used for generating steady states (Ahmad, 2009). This tool facilitates the discrete modeling of a BRN. The discrete modeling formalism has been implemented in this tool. A part from the discrete modeling, this tool also facilitate in the analysis of stead state behaviors (cycles and stable states).

BRNs can have both positive and negative feedback loops, where positive loops are the necessary conditions for stable states and negative loops are the necessary conditions for homeostasis. The model in current study only has negative feedback loop. Two types of variables are used in model building: one is by using Boolean values (0 and 1) and second is by using multi-valued or kinetic logic (starting from 2 to any value). In figure 2.6 we used Boolean values interpreting that ATM is sending weak signal to p53 whereas figure 2.7 is showing multi-valued model, strong signal of ATM for p53 phosphorylation. These different logics will not affect the parameters (Table 2.1) but it will change the overall result.

2.5. Graphical view of Model

Graphiz (software) package contains graph visualization software. Graph visualization is a way of representing structural information as diagram of abstract network and graph graphviz-2.26.3 is used for the graph generation and analysis and from these graph cycle and deadlock state is easily analyzed.

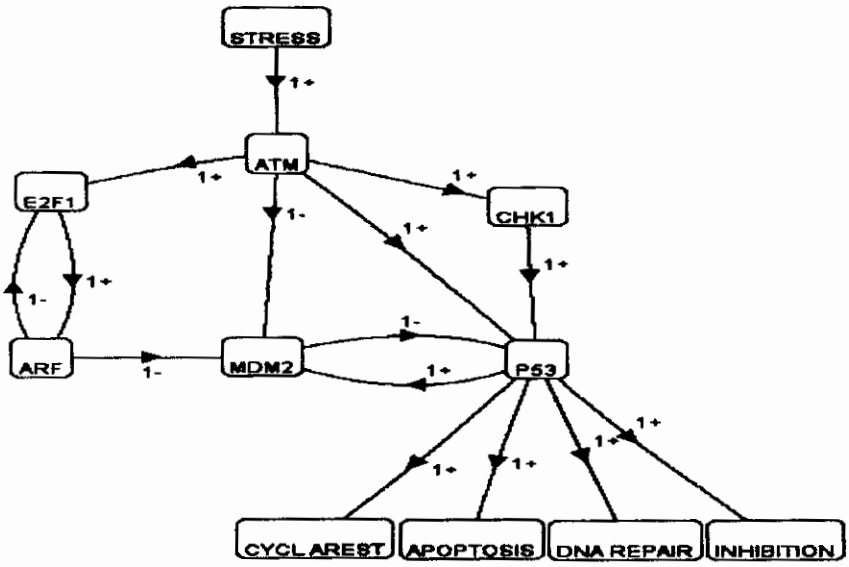


Figure 2.5: Genotech model of p53 associated regulatory network

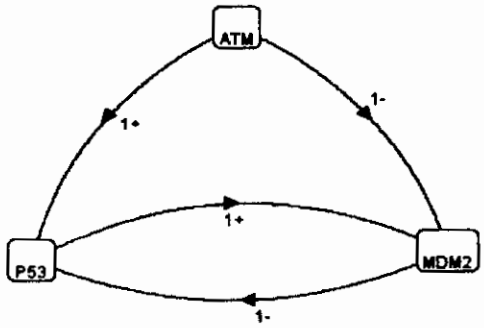


Figure 2.6: Abstracted p53 associated BRN (Boolean logic)

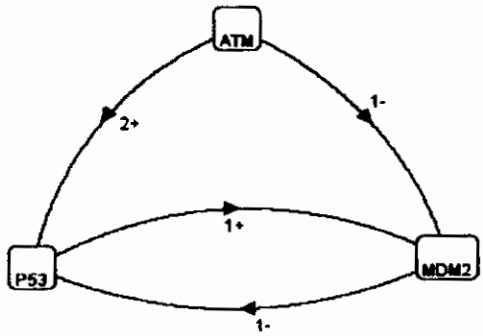


Figure 2.7: Abstracted p53 associated BRN (Multi-valued or kinetic logic)

Table 2.1: Logical parameters for the BRN

Protein	Activator	Inhibitor	Parameters
ATM	NILL	NILL	K (ATM , { }) = 0
p53	ATM	Mdm2	K (p53, {}) = 0 K (p53, {ATM}) = 1 K (p53, {Mdm2}) = 1 K (p53, {ATM,Mdm2}) = 1
Mdm2	P53	ATM	K (Mdm2,{}) = 0 K (Mdm2,{ATM}) = 1 K (Mdm2,{p53}) = 1 K (Mdm2,{ATM,p53}) = 1

Table 2.2: Logical parameters for the BRN

Protein	Activator	Inhibitor	Parameters
ATM	NILL	NILL	K (ATM , { }) = 0
p53	ATM	Mdm2	K (p53, {}) = 0 K (p53, {ATM}) = 1 K (p53, {Mdm2}) = 1 K (p53, {ATM,Mdm2}) = 1
Mdm2	P53	ATM	K (Mdm2,{}) = 0 K (Mdm2,{ATM}) = 1 K (Mdm2,{p53}) = 1 K (Mdm2,{ATM,p53}) =0

2.6. Hybrid Modeling

After obtaining the right set of parameters, the 2nd step of Dynamical Modeling was applied, i.e., Hybrid Modeling.

Hybrid systems bring together both discrete and continuous components normally controllers having interaction with the physical environment. In Hybrid modeling both discrete and continuous features were employed to study diverse biological properties and to explore several biological properties in detail (Ahmad *et al.,* 2009).

For Hybrid systems specification analysis and verification methods are required as there is a growing mass of embedded applications particularly in safety critical areas, e.g., avionics or automotive electronics. For automatic verification for specific subclasses, Hybrid automata have been designed entailing discrete as well as continuous specification method (Uller and Stauner, 1996).

In order to handle with temporal issues in a biological phenomenon that is a continuous non-linear process in contrast with discrete data, Hybrid modeling has been used. In this modeling formalism the sigmoid-curve is no longer discretised, rather a piece-wise linear curve would be developed. Here the delays required for gene evolution are represented in terms of time intervals and clocks (Ahmad *et al.,* 2009). Hybrid systems are generally known as timed automaton formalism (Alur and Dill, 1994).

2.6.1. Clock

A clock is a vector of continuous variable. A clock is associated with each gene, i.e., synchronous rate of evolution with respect to time for each gene will be recorded. At

each transition these clocks record the rate of evolution such that whenever the system passes from one location to the other, clocks will be reset to 0 (Ahmad *et al.,* 2009). In case of Linear Hybrid Automata (LHA) also applies time period in terms of clock (Henzinger and Ho, 1995). The current value of a clock represents the time elapsed in the discrete space to cover up the latest transition.

2.6.2. Linear Hybrid Automata

A famous modeling formalism exhibiting both discrete and continuous behavior is called Hybrid automaton (HA) (Alur *et al.,* 1995; Lynch *et al.,* 2003). It is a modified version of finite state automaton in which discrete states are matched/ linked to continuous dynamics of a system. In a HA, the discrete and continuous, both types of variables were used to represent the state of the system. In a dynamical system the continuous variables evolve when it reaches a certain threshold level at the same time then the discrete variable shift to new value. Then in the new state, the continuous variable starts to evolve again (Antoniotti *et al.,* 2003). Embedded systems are modeled using HA which includes automated highway systems, air traffic management, embedded automotive controllers, robotics and real time circuits. HA is being used recently to model and analyze BRN such as bio-molecular networks (Alur *et al.,* 2001), metabolic processes (Hespanha and Singh, 2005; Lincoln and Tiwari, 2004), PSPs (Ghosh and Tomlin, 2004) and immune response (Tieri *et al.,* 2005). BRNs are fundamentally hybrid in nature: biochemical concentrations continuously vary in nature it is possible they can be discretized by using distinct states.

LHA is a finite sequence of 6 terms ($l, l_0, X, P, Œ, \dot{I}nv, Pif$) where,

- A fixed set of locations denoted by l

- l_0 is a part of l representing initial location
- X is a fixed number of clocks
- Delay constraints in a finite set are denoted by P
- Finite set of edges $\varepsilon = (l, g, \square, l') \in \mathcal{E}$, it represents an edge has a guard g and it transmit from l to l' and the set reset $K \subseteq X$
- Inv: work is to allocate immutable to a location
- Pif: $l \times X \rightarrow \{-1,0,1\}$, mapping of evolution rate with each pair (l, n)

2.6.3. Period

The results obtained from HYTECH analysis requires the definition of full period (denoted by π (p)) as shown in figure 2.8. It is the sum of all the delays (once at each expression level) that any gene goes through sequentially. The gene's original round can be greater than the full time period as there can be lazy phases in gene expression (with no increase or decrease) (Ahmad, 2009). The results obtained from HYTECH were expressed as constraints, showing the nature of the cycle.

2.6.4. Invariance Kernel

An interesting area of mathematics is viability theory. A system execution is considered as viable only if the trajectories of the system remain within a set (viability domain).the set is called invariant set and the largest of this set is called invariance kernel. Invariance kernel gives information about the behavior of the cycle. It is a set of states which generate a trajectory by primordial permissible command and then remain in it by satisfying the constraints forever. In a BRN, let all the temporal state

space is given by a subset K_0. When $x \in K_0$, then set of K_0 is immutable it says that every trajectory or pathway starting in x is feasible and executable in K_0. The largest unalterable subset of K_0 is known as invariance kernel. If the system comes outside the kernel then it will move in divergence trajectories leading to stable steady states (Ahmad and Roux, 2009).

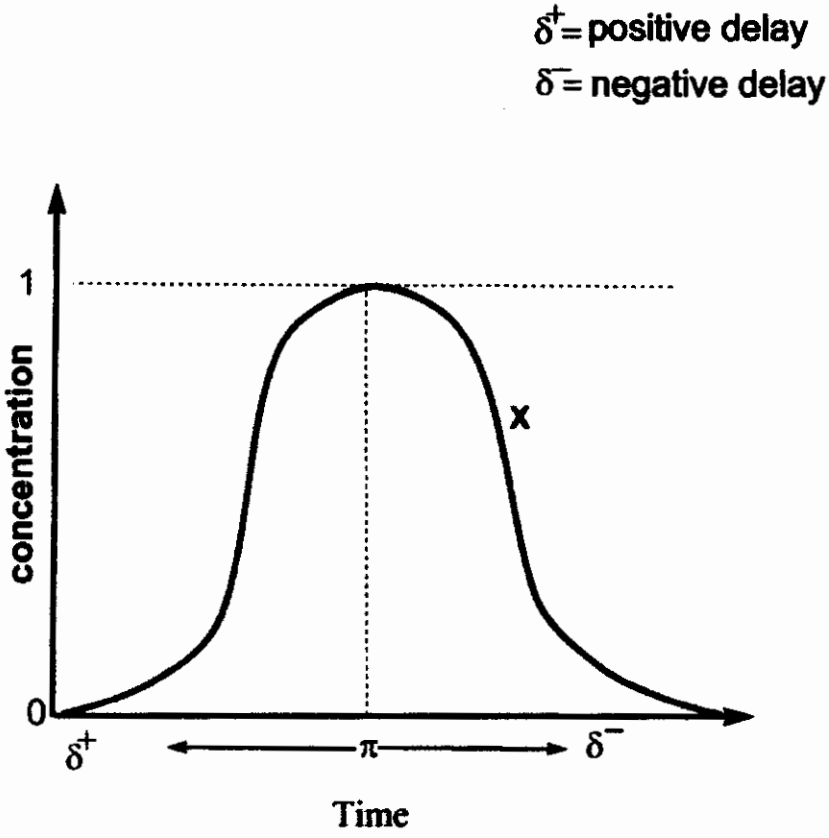


Figure 2.8: Figure showing the period for gene X

CHAPTER 3

RESULTS

3.1. Dynamical Modeling Results of P53 Associated BRN

A broad collection of diverse modeling approaches have been reported which can be used to imitate the performance of BRNs and all of the resulting models have some similarities in their approach and abstractions. Dynamical modeling is also one of these modeling approaches. Its focal function is to unfold and simulate the behavior and change in the behavior of the state of the system. In case of BRNs, state of the system represents the level of concentration of regulatory components and change in the state of the system would be use to represent change in the concentration of the regulatory factors as a result of biological process such as transcription, translation or decay events. The key aspects of a dynamical formalism is to provide detail description of system's states in term of time and systematic view of temporal states and how these states are linked. The elements used in dynamical formalism give us states, state space and transitions (Geard and Willadse, 2009). Steady states or stable oscillations are the most prominent features of a state space; they could be either normal equilibrium state of the system or the abnormal (diseased) state of the system (Kaufman, 1969; Huang, 2004).

The results generated by the HYTECH tool have been shown in the form of a discretization map. This discretization of kinetic parameters corresponding to the logical parameters reveals valuable information about the characteristics of the BRN as discussed below with respect to the p53 activation in case of stress.

3.2. State Table

The parameter optimization leads the model to generate a state table as depicted in table 2.1 and 2.2. The state tables illustrated in tables 3.1, 3.2 and 3.4 in addition to

Input and their respective Output states (transition states), also contains Weight (w) and K values. w represents weight of the input state while k shows maximum activation level of a protein, after applying the weight (w). The initial state is given in light green colour and the epigenetic state (Deadlock state) is coloured Red in state table (Table 3.1). A state generally has (n-1) output states where 'n' is the number of elements (proteins) involved in the system. Three different observations has been made by using Boolean and multi-valued model given in figure 2.6 and 2.7, and logical parameters given in table 2.1 and 2.2.

- Table 3.1 has been generated by applying logical parameters given in table 2.2 on Boolean model given in figure 2.6 will be further referred as **1st Model**
- Table 3.1 has been generated by applying logical parameters given in table 2.1 on multi-valued model given in figure 2.7 will be further referred as **2nd Model**
- Table 3.1 has been generated by applying logical parameters given in table 2.2 on multi-valued model given in figure 2.7 will be further referred as **3rd Model**

In the state tables over here, (0, 0, 1) represents the normal state (stable steady state) of unstressed cells in which transcription factor Mdm2 is active (shown in table 3.1, 3.2 and 3.3). State (2, 0, 1) is considered as the initial state of the system (1, 0, 1 in case of 1st model), when cell has sensed the stress and ATM become activated (shown in table 3.1, 3.2 and 3.3). State (0, 1, 0) is again a stable steady state depict the over expression of p53 (shown in table 3.1 and 3.3), resulting in the development of cancer.

Table 3.1: State Transition table of Boolean model (1st Model)

Proteins /Genes			Weights			K Parameters			Transition States
ATM	P53	MDM2	Watm	Wp53	Wmdm2	Katm	Kp53	Kmdm2	
0	0	0	{}	{MDM2}	{ATM}	0	1	1	[[0, 1, 0], [0, 0, 1]]
0	0	1	{}	{}	{ATM}	0	0	1	[ ]
0	1	0	{}	{MDM2}	{ATM, P53}	0	1	0	[ ]
0	1	1	{}	{}	{ATM, P53}	0	0	0	[[0, 0, 1],[0, 1, 0]]
1	0	0	{}	{ATM, MDM2}	{}	0	1	0	[[0, 0, 0], [1, 1, 0]]
1	0	1	{}	{ATM}	{}	0	1	0	[[0, 0, 1], [1, 1, 1], [1, 0, 0]]
1	1	0	{}	{ATM, MDM2}	{P53}	0	1	1	[[0, 1, 0], [1, 1, 1]]
1	1	1	{}	{ATM}	{P53}	0	1	1	[[0, 1, 1]]

Table 3.2: State Transition table of multi valued model (2nd model)

Proteins /Genes			**Weights**			**K Parameters**			**Transition States**
ATM	**P53**	**MDM2**	**Watm**	**Wp53**	**Wmdm2**	**Katm**	**Kp53**	**Kmdm2**	
0	0	0	{}	{MDM2}	{ATM}	0	1	1	[[0, 1, 0], [0, 0, 1]]
0	0	1	{}	{}	{ATM}	0	0	1	[ ]
0	1	0	{}	{MDM2}	{ATM, P53}	0	1	1	[[0, 1, 1]]
0	1	1	{}	{}	{ATM, P53}	0	0	1	[[0, 0, 1]]
1	0	0	{}	{MDM2}	{}	0	1	0	[[0, 0, 0], [1, 1, 0]]
1	0	1	{}	{}	{}	0	0	0	[[0, 0, 1], [1, 0, 0]]
1	1	0	{}	{ MDM2}	{P53}	0	1	1	[[0, 1, 0], [1, 1, 1]]

1 1 1	{} {} {P53}	0 0 1	[[0, 1, 1], [1, 0, 1]]
2 0 0	{} {ATM, MDM2} {}	0 0	[[1, 0, 0]]
2 0 1	{} {ATM} {}	0 1 0	[[1, 0, 1], [2, 1, 1], [2, 0, 0]]
2 1 0	{} {ATM, MDM2} {P53}	0 1	[[1, 1, 0], [2, 1, 1]]
2 1 1	{} {ATM} {P53}	0 1 1	[[1, 1, 1]]

Table 3.3: State Transition table multi valued model (3rd model)

Proteins /Genes			Weights			K Parameters			Transition States
ATM	P53	MDM2	Watm	Wp53	Wmdm2	Katm	Kp53	Kmdm2	
0	0	0	{}	{MDM2}	{ATM}	0	1	1	[[0, 1, 0], [0, 0, 1]]
0	0	1	{}	{}	{ATM}	0	0	1	[ ]
0	1	0	{}	{MDM2}	{ATM, P53}	0	1	0	[ ]
0	1	1	{}	{}	{ATM, P53}	0	0	0	[[0, 0, 1], [1, 1, 0]]
1	0	0	{}	{MDM2}	{}	0	1	0	[[0, 0, 0], [1, 1, 0]]
1	0	1	{}	{}	{}	0	0	0	[[0, 0, 1], [1, 0, 0]]

1 1 0	{} { MDM2} {P53}	0 1 1	[[0, 1, 0], [1, 1, 1]]
1 1 1	{} {} {P53}	0 0 1	[[0, 1, 1], [1, 0, 1]]
2 0 0	**{} {ATM, MDM2} {}**	**0 1 0**	**[[1, 0, 0], [2, 1, 0]]**
2 0 1	{} {ATM} {}	0 1 0	[[1, 0, 1], [2, 1, 1], [2, 0, 0]]
2 1 0	**{} {ATM, MDM2} {P53}**	**0 1 1**	**[[1, 1, 0], [2, 1, 1]]**
2 1 1	**{} {ATM} {P53}**	**0 1 1**	**[[1, 1, 1]]**

3.3. State graph

A state graph gives a straight forward visualization of all states of a system towards subsequent states. The state graphs were obtained by using the same order of logical parameters and BRNs as used for state tables (section 3.1). Two different type of graph has been generated; one by using the software GENOTECH and then software GRAPHIZ has been used for more clarity of graph and elaborate explanation of steady states. In the figures generated by using the GRAPHIZ, order of proteins was given according to the setting of parameters, highlighted at the top-left corner. The Stable Steady State can be easily identified (coloured in red) having no outgoing while the one cycles was also apparent in this state graph. Two stable steady states were observed one represents the diseased condition whereas other stable steady state represents the condition of normal unstressed cell. The cyclic state however represents the haemostatics which should be maintained in response of stress for the activation of downstream events such as cell cycle arrest, apoptosis etc.

3.4. Homeostasis of p53 associated BRN

The p53 BRN show normal and healthy function when it is regulated in a cyclic form in response of stress. In Figure 3.4 and 3.6 a cyclic behavior of p53 and its transcription factor Mdm2 is oberved (cycle in figure 3.7). The system moved in a cyclic behavior when ATM is in active form and ATM is activated only as result of DNA damage or some stress. René Thomas formalism predicts cycle as oscillation in the behavior of system or homeostasis as there is negative feedback loop in Mdm2 and p53.

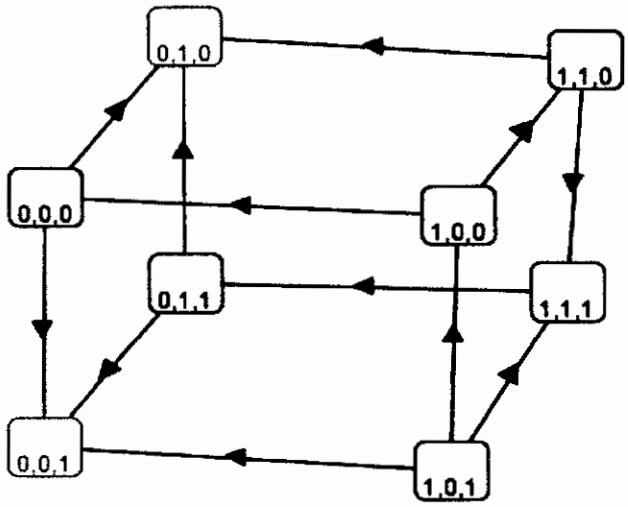


Figure 3.1: GENOTECH generated graph of p53 associated Boolean model

(1st Model)

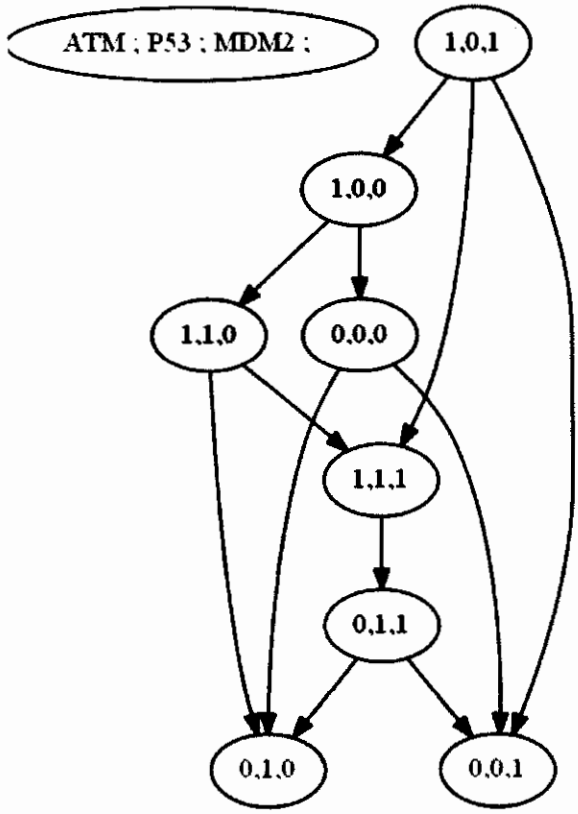


Figure 3.2: Systematic diagram showing all possible paths of p53 Mdm2 and ATM (1st Model)

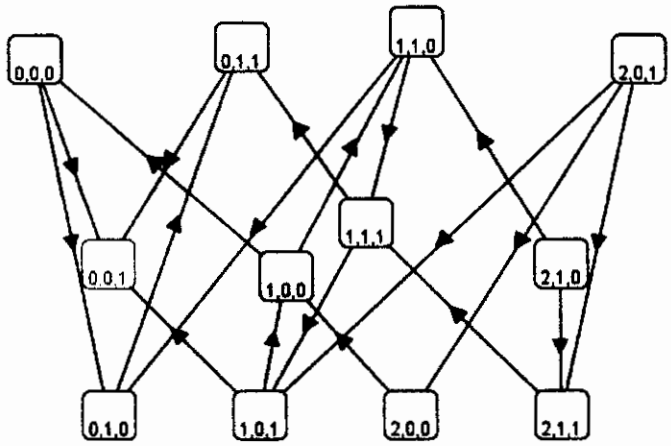


Figure 3.3: GENOTECH generated graph of p53 associated multi valued model (2^{nd} Model)

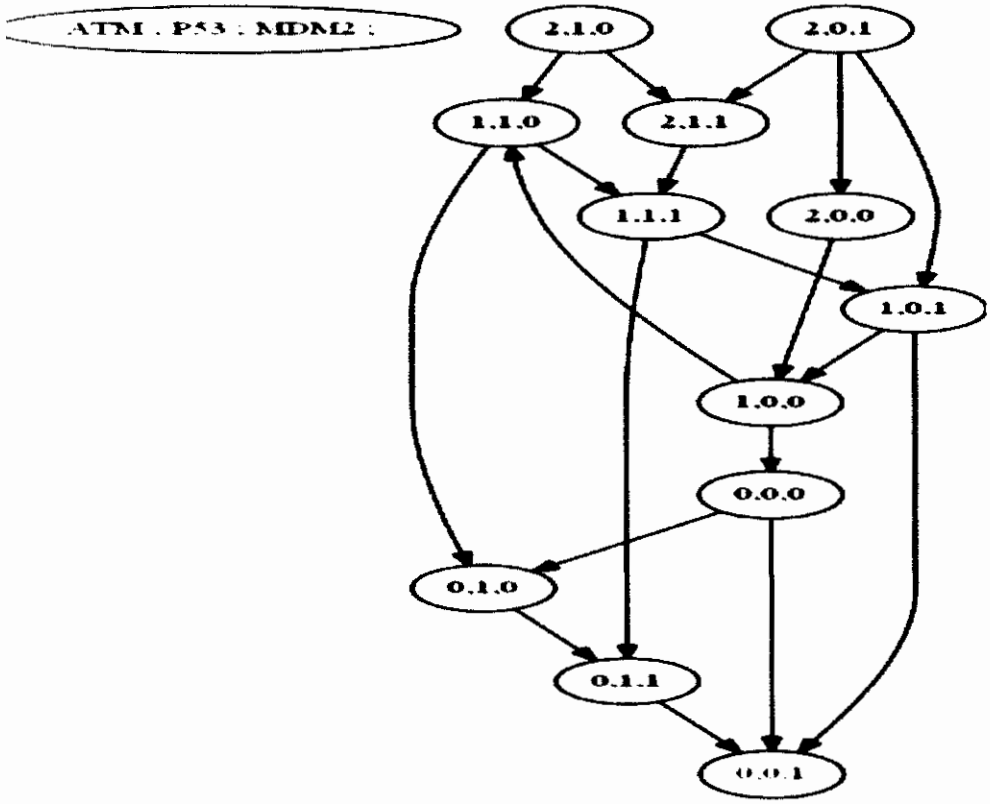


Figure 3.4: Systematic diagram showing all possible paths of p53 Mdm2 and ATM and cyclic behavior of p53 and Mdm2 (2nd Model)

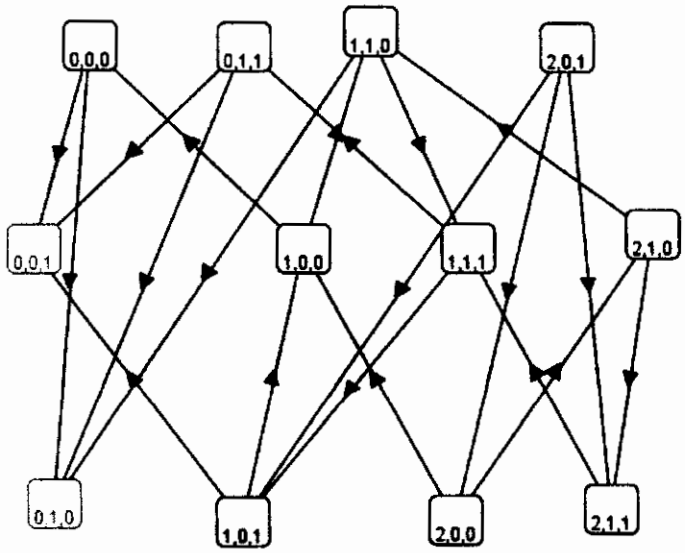


Figure 3.5: GENOTECH generated graph of p53 associated Boolean model (3rd Model)

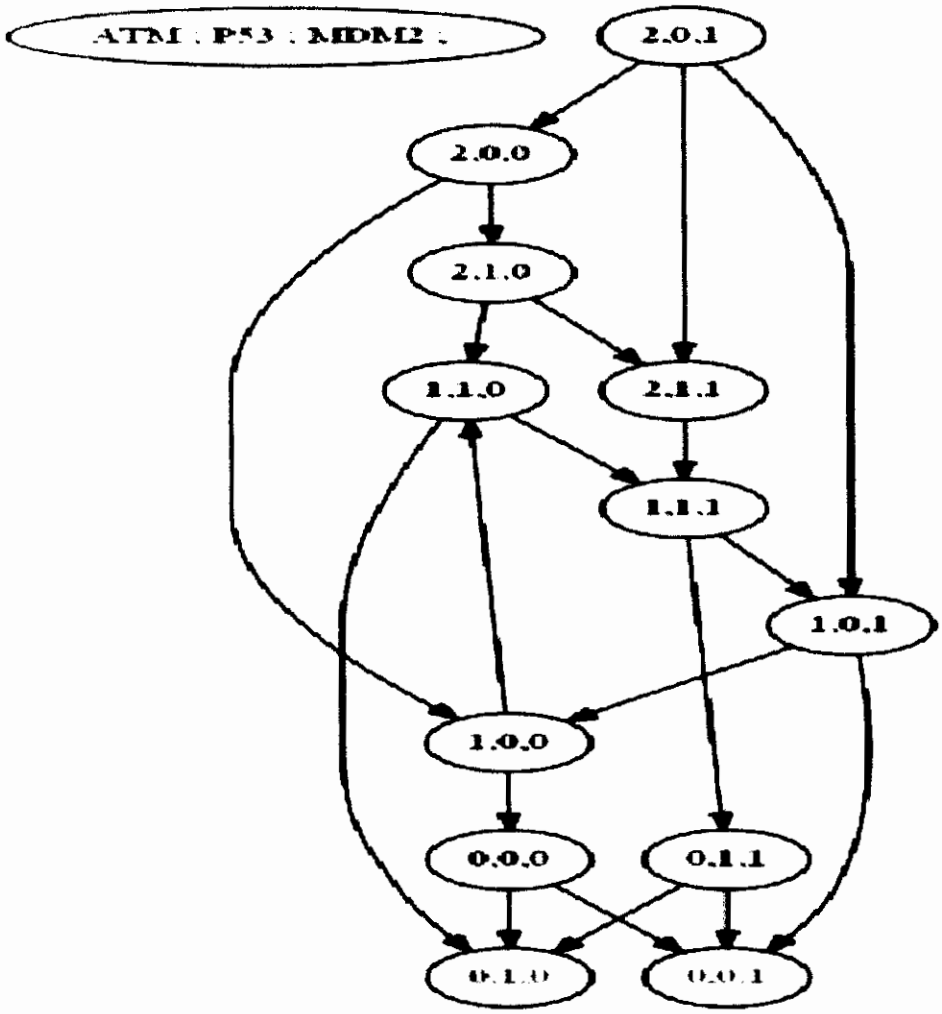


Figure 3.6: Systematic diagram showing all possible paths of ATM, p53 and Mdm2 and cyclic behavior of p53 and Mdm2 (3rd Model)

Each state in the graph has three elements in it ATM which is only phosporylated in the case of stress, p53 and its inhibitor Mdm2. The cycle that is generated by using René Thomas model is:

$$\{(1, 0, 0) \rightarrow (1, 1, 0) \rightarrow (1, 1, 1) \rightarrow (1, 0, 1) \rightarrow (1, 0, 0)\}$$

The state of the system is defined in terms of discrete variables that take the logical values 0, 1 and 2. The initial condition of the cycle is supposed to be (1, 0, 1), ATM has sensed the stress and become phosporylated. Active ATM inhibits the Mdm2 and activates the p53, states become (1, 0, 0) and (1, 1, 0) respectively. We can also explain this transition by saying that activated ATM has inhibited the Mdm2 and inhibition of Mdm2 results into the activation of p53 as Mdm2 is transcription factor of p53. Activated p53 again up regulates the Mdm2 and states become (1, 0, 1) and whole cycle is repeated.

They cyclic behavior of regulatory network is not observed in case of Boolean model. Which can be interpreted as stress signal is not strong enough to maintain the homeostasis in the system (as shown in figure 3.2).

3.5. Stable steady states in p53 associated BRN

The discrete modeling highlights the effects of threshold to discretise the concentration and the dynamics in the modeling depend on the discrete parameters called the attractors or targets. Different possible paths and states have been generated in the graph, which are helpful in predicting the behavior of the system.

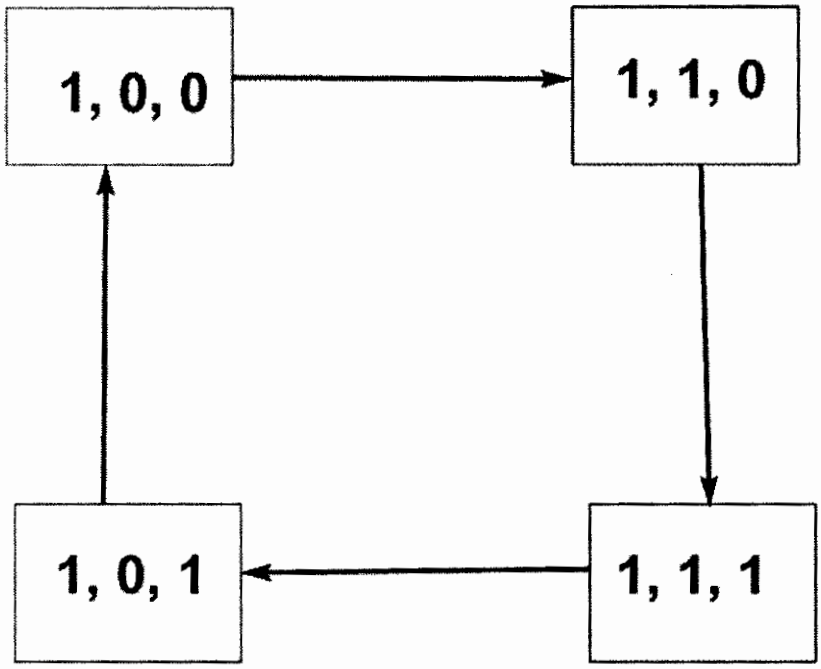


Figure 3.7: Cycle representing homeostasis of the BRN

Two stable steady states are obvious in figure 3.6 and 3.2 while in figure 3.4 only one stable state is evident. The reason of this difference in the graphs is parameter optimization. The state graphs or state tables, in which two stable steady states are observed their K parameter has been changes as follow:

K (Mdm2, {ATM, p53}) = 1is replaced by the parameter K (Mdm2, {ATM, p53}) =0

This parameter has been changed by assuming that in case of stress the interaction between p53 and Mdm2 became stronger then the positive interaction between p53 and Mdm2.In case of stress the rate of inhibition of Mdm2 is higher as compare to the production of Mdm2.

The steady state is (0, 0, 1) is showing equilibrium condition, means that ATM and p53 are both at 0 level while Mdm2 is at high level. This stable steady state is showing the normal condition of cell in the absence of stress or after responding the stress. Once the stress signal has diminished, the system should have entered into this state in order to perform normal body's function. The other stable steady state (0, 1, 0) is the diseased condition, showing the expression of p53 only. P53 should be only expressed in cyclic manner and in the presence of ATM (stress). This stable steady state is the one where p53 does not perform its normal function of DNA reparation, cell cycle arrest and apoptosis. From initial states there are many pathways that lead to steady states as described in next section.

3.5.1. Channel towards Stable Steady State

Many transitions exist at each state. There are many pathways exists moving from initial state toward dead lock state. A list of some pathways is shown in tables given below.

In Table 3.4, 3.5 and 3.6, list of some pathways is given moving from initial state to deadlock state for all three models. The states mention in the red are the states representing the stable steady states either diseased or normal. First index of all three tables are exhibiting the shortest pathway the BRN can adopt to reach the output state. First index show the transition state behaviour towards the output state which is achieved without entering into the whole cyclic behaviour. All possible pathways obtained from all three models are given in tables. Some indices of the tables are showing the path way which enter in the cycle and then diverge toward the stead stable states for example indices 7, 8, 12, 13, 18 and 19 in table 3.5 and 13, 14 and 18 in table 3.6 are showing the same pathways.

3.5.2. Divergence from cycle

Figure 3.8 is showing some of the pathways from 3rd model in which the paths are showing divergence from the cyclic states of the system and moves toward the steady states either normal or diseased. Figure 3.8 a & b showing that after exiting the cycle the pathway moves to another unstable steady state of the system, at that state there is equal chance of entering in the diseases state or normal state of the system. Figure 3.8 c and d depicting that after leaving the cycle the path directly jumps in to the stable steady states either to disease that is 0, 1, 0 or normal 0, 0, 1.

Table 3.4: Possible Pathways in 1st Model leading toward Stable Steady States

Index	Pathway to Stable Steady State
1.	1,0,1 ⟶ 0, 0, 1
2.	1,0,1 ⟶ 1,1,1 ⟶ 0,1,1 ⟶ 0, 0, 1
3.	1,0,1 ⟶ 1,1,1 ⟶ 0,1,1 ⟶ 0, 1, 0
4.	1,0,1 ⟶ 1,0,0 ⟶ 0,0,0 ⟶ 0, 1, 0
5.	1,0,1 ⟶ 1,0,0 ⟶ 0,0,0 ⟶ 0, 0, 1
6.	1,0,1 ⟶ 1,0,0 ⟶ 1,1,0 ⟶ 0, 1, 0
7.	1,0,1 ⟶ 1,0,0 ⟶ 1,1,0 ⟶ 1, 1, 1 ⟶ 0, 1, 1 ⟶ 0, 1, 0
8.	1,0,1 ⟶ 1,0,0 ⟶ 1,1,0 ⟶ 1, 1, 1 ⟶ 0, 1, 1 ⟶ 0, 0, 1

Table 3.5: Possible Pathways in 2nd Model leading toward Stable Steady States

Index	Pathway to Stable Steady State
1.	2, 0, 1 ⟶ 1,0,1 ⟶ 0, 0, 1
2.	2,0,1 ⟶ 2,0,0 ⟶ 1,0,0 ⟶ 0, 0, 0 ⟶ 0, 0, 1
3.	2,0,1 ⟶ 2, 1,1 ⟶ 1,1,1 ⟶ 0,1,1 ⟶ 0, 0, 1
4.	2, 0, 1 ⟶ 1,0,1 ⟶ 1, 0, 0 ⟶ 0, 0, 0 ⟶ 0, 0, 1
5.	2,0,1 ⟶ 2, 1,1 ⟶ 1,1,1 ⟶ 1,0,1 ⟶ 0, 0, 1
6.	2, 0, 1 ⟶ 1,0,1 ⟶ 1, 0, 0 ⟶ 0, 0, 0 ⟶ 0, 1, 0 ⟶ 0, 1, 1 ⟶ 0, 0, 1
7.	2,0,1 ⟶ 2, 1,1 ⟶ 1,1,1 ⟶ 1,0,1 ⟶ 1, 0, 0 ⟶ 0, 0, 0 ⟶ 0, 1, 0 ⟶ 0, 1, 1 ⟶ 0, 0, 1
8.	2,0,1 ⟶ 2, 1,1 ⟶ 1,1,1 ⟶ 1,0,1 ⟶ 1, 0, 0 ⟶ 0, 0, 0 ⟶ 0, 0, 1
9.	2,0,1 ⟶ 2, 1,1 ⟶ 1,1,1 ⟶ 0,1,1 ⟶ 0, 0, 1

10.	2,0,1 ⟶ 2, 0, 0 ⟶1,0,0 ⟶0, 0,0⟶ 0, 1, 0 ⟶0, 1, 1⟶ 0, 0, 1
11.	2, 1,0⟶ 2,1,1⟶ 1,1,1⟶0, 1, 1⟶ 0, 0, 1
12.	2,1,0 ⟶2, 1,1⟶ 1,1,1⟶ 1, 0, 1⟶ 1, 0, 0⟶0, 0, 0⟶ 0, 0, 1
13.	2,1,0⟶ 2, 1,1⟶ 1,1,1⟶ 1, 0, 1 ⟶ 1, 0, 0⟶0, 0, 0 ⟶ 0, 1, 0⟶0, 1, 1 ⟶ 0, 0, 1
14.	2, 1,0⟶ 2,1,1⟶ 1,1,1⟶1, 0, 1⟶ 0, 0, 1
15.	2,1,0 ⟶1,1,0 ⟶ 0, 1, 0⟶ 0, 1, 1⟶0, 0, 1
16.	2,1,0 ⟶1,1,0⟶1,1,1⟶1, 0, 1⟶0, 0, 1
17.	2,1,0 ⟶1,1,0⟶1,1,1 ⟶ 0, 1, 1⟶0, 0, 1
18.	2,1,0 ⟶1,1,0⟶1,1,1⟶1, 0, 1⟶1, 0, 0⟶ 0, 0, 0⟶ 0, 1, 0⟶0, 1, 1⟶ 0, 0, 1
19.	2,1,0 ⟶1,1,0⟶1,1,1⟶1, 0, 1⟶1, 0, 0⟶ 0, 0, 0⟶ 0, 0, 1

Table 3.6: Possible Pathways in 3rd Model leading toward Stable Steady States

Index	Pathway to Stable Steady State
1.	2, 0, 1 ⟶ 1, 0, 1 ⟶ 0, 0, 1
2.	2, 0, 1 ⟶ 1, 0, 1 ⟶ 1, 0, 0 ⟶ 0, 0, 0 ⟶ 0, 0, 1
3.	2, 0, 1 ⟶ 1, 0, 1 ⟶ 1, 0, 0 ⟶ 0, 0, 0 ⟶ 0, 1, 0
4.	2, 0, 1 ⟶ 2, 1, 1 ⟶ 1,1, 1 ⟶ 0,1,1 ⟶ 0, 0, 1
5.	2, 0, 1 ⟶ 2, 1, 1 ⟶ 1, 1, 1 ⟶ 0, 1, 1 ⟶ 0, 1,0
6.	2, 0, 1 ⟶ 2, 1, 1 ⟶ 1, 1, 1 ⟶ 1, 0, 1 ⟶ 0, 0, 1
7.	2, 0, 1 ⟶ 2, 1, 1 ⟶ 1, 1, 1 ⟶ 1, 0, 1 ⟶ 0, 1, 0
8.	2, 0, 1 ⟶ 2, 0, 0 ⟶ 1, 0, 0 ⟶ 0, 0, 0 ⟶ 0, 0, 1
9.	2, 0, 1 ⟶ 2, 0, 0 ⟶ 1, 0, 0 ⟶ 0, 0, 0 ⟶ 0, 1, 0

10.	2, 0, 1 → 2, 0, 0 → 2, 1, 0 → 1, 1, 0 → 0, 1, 0
11.	2, 0, 1 → 2, 0, 0 → 0, 0, 0 → 0, 1, 0
12.	2, 0, 1 → 2, 0, 0 → 2, 1, 0 → 1, 1, 0 → 1, 1, 1 → 1,0,1 → 0, 1, 0
13.	2, 0, 1 → 2, 0, 0 → 2, 1, 0 → 1, 1, 0 → 1, 1, 1 → 1,0,1 → 1, 0, 0 → 0, 0, 0 → 0, 0, 1
14.	2, 0, 1 → 2, 0, 0 → 2, 1, 0 → 1, 1, 0 → 1, 1, 1 → 1,0,1 → 1, 0, 0 → 0, 0, 0 → 0, 1, 0
15.	2, 0, 1 → 2, 0, 0 → 2, 1, 0 → 1, 1, 0 → 0, 1, 0
16.	2, 0, 1 → 2, 0, 0 → 2, 1, 0 → 1, 1, 0 → 1, 1, 1 → 1,0,1 → 0, 0, 1
17.	2, 0, 1 → 2, 0, 0 → 2, 1, 0 → 1, 1, 0 → 1, 1, 1 → 1,0,1 → 0, 1, 0
18.	2, 0, 1 → 2, 0, 0 → 2, 1, 0 → 1, 1, 0 → 1, 1, 1 → 1,0,1 → 1, 0, 0 → 0, 0, 0 → 0, 1, 0

19.	2, 0, 1 → 2, 0, 0 → 2, 1, 0 → 1, 1, 0 → 1, 1, 1 → 1,0,1 → 1, 0, 0 → 0, 0, 0 → 0, 0, 1
20.	2, 0, 1 → 2, 0, 0 → 2, 1, 0 → 2, 1, 1 → 1, 1, 1 → 0 ,1,1 → 0, 1, 0
21.	2, 0, 1 → 2, 0, 0 → 2, 1, 0 → 2, 1, 1 → 1, 1, 1 → 0 ,1,1 → 0, 0, 1
22.	2, 0, 1 → 2, 0, 0 → 2, 1, 0 → 2, 1, 1 → 1, 0, 1 → 0, 0, 1
23.	2, 0, 1 → 2, 0, 0 → 2, 1, 0 → 2, 1, 1 → 1, 0, 1 → 1, 0, 0 → 0, 0, 0 → 0, 1, 0
24.	2, 0, 1 → 2, 0, 0 → 2, 1, 0 → 2, 1, 1 → 1, 0, 1 → 1, 0, 0 → 0, 0, 0 → 0, 0, 1

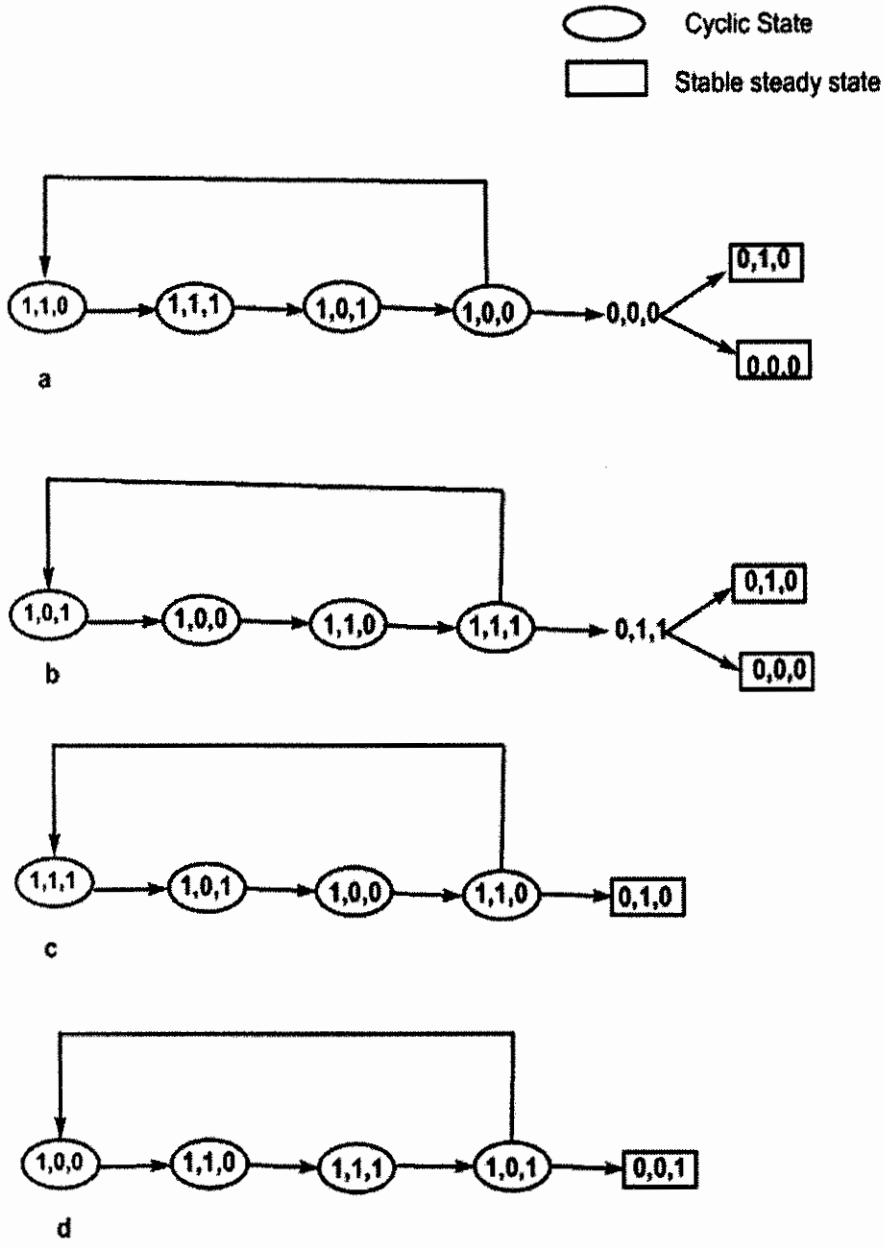


Figure3.8: Systematic representation of pathways showing deviated from the cycle

3.6. Real Time Modeling Result of p53 Associated BRN

GENOTECH has been used to find the states of the system. Further Real time model of the system will be obtained by introducing the time delays for these states. **Hytech** software will be used to introduce the time delays in the system. The introduction of the time delays will be helpful for the system in order to decide whether to stay in the cycle or to move to the other states in the space leading toward the deadlocks (either normal or disease).

The delay constrains form each state and for the whole path way is obtained for 3rd model only, the graph of which is given in figure 3.6. This is because the model is showing the combine result from all three different experiments. Two stable steady states or dead lock states (diseased as well as normal) and cycle are clear in the graph. The invariance kernel has been obtained for both 2nd and 3rd Model.

3.6.1. Time Delays of Pathways leading to Stable Steady State

The time constraints of each state towards the output state are given in Table 3.6. All the states are enlisted here as some of the states are neither involved in the path of cycle nor in the path leading towards deadlock state as traverse from the one initial state. The delays are given in the form of equations in which δ represent change of, '+' or '-' sign represent activation and degradation respectively and base value of δ represents the element and its location from where it start evolving. The delay of first index shows that,

$$\mathbf{201 \rightarrow 200} = |\delta^{-}_{MDM2201}| \leq |\delta^{-}_{ATM201}|$$
$$|\delta^{+}_{MDM2201}| \leq \delta^{+}_{P53201}$$

The states move from 201 toward 200, the system is activated and degradation of Mdm2 has started. Its degradation will start when negative time delay $\delta^{-}_{MDM2201}$ of Mdm2 i.e degradation of Mdm2 protein, is less than or equal to negative time delay δ^{-}_{ATM201} of ATM or positive time delay δ^{+}_{P53201} of p53.

The delays can also be in a complex form, e.g.

$$100 \rightarrow 000 = |\delta^{-}_{ATM100}| \leq \delta^{+}_{P53201} + \delta^{-}_{MDM2101}$$

It shows that degradation or activation rate not only depends on input states but its concentration build up or degradation can be start at previous states. In the above equation one can see that time delay for the degradation of ATM at 100 should be less than or equal to the positive delay of p53 at initial state of the system 201 and also it should be less than or equal to negative time delay of Mdm2 at the previous state 101.

On the basis of the information given in Table 3.6 state transitions trajectories can be designed for favorable pathways and it will also give the source for controlling the desired state transition using these constraints

3.6.2. Time Delays for the whole pathway

Table 3.8 is identical to Table 3.6, it has addition of one more feature i.e. delay to each transition and has state transition as well.

$$201 \xrightarrow{\delta^{-}_{ATM201} \leq \delta^{-}_{MDM2201}} 101 \xrightarrow{\delta^{-}_{ATM101} \leq \delta^{-}_{MDM2101}} \mathbf{001}$$

Table 3.8: Pathways with Time Delays Moving towards Deadlock State

Index	Pathway towards Deadlock states
1	$201 \xrightarrow{\delta^-_{ATM201} \le \delta^-_{MDM2201}} 101 \xrightarrow{\delta^-_{ATM101} \le \delta^-_{MDM2101}} \mathbf{001}$
2	$201 \xrightarrow{\delta^-_{ATM201} \le \delta^-_{MDM2201}} 101 \xrightarrow{\delta^-_{MDM2101} \le \delta^-_{ATM201}} 100 \xrightarrow{\lvert\delta^-_{ATM100}\rvert \le \delta^+_{P53201} + \delta^-_{MDM2101}} 000 \xrightarrow{\delta^+_{MDM2000} \le \delta^-_{P53000}} \mathbf{001}$
3	$201 \xrightarrow{\delta^-_{ATM201} \le \delta^-_{MDM2201}} 101 \xrightarrow{\delta^-_{MDM2101} \le \delta^-_{ATM201}} 100 \xrightarrow{\lvert\delta^-_{ATM100}\rvert \le \delta^+_{P53201} + \delta^-_{MDM2101}} 000 \xrightarrow{\delta^+_{P53000} \le \delta^+_{MDM2000}} \mathbf{010}$
4	$201 \xrightarrow{\delta^+_{P53201} \le \delta^-_{MDM2201} + \delta^-_{ATM201}} 211 \xrightarrow{\lvert\delta^-_{ATM211}\rvert \le \lvert\delta^-_{P53111}\rvert + 2\delta^+_{P53201} + \delta^-_{MDM2111}} 111 \xrightarrow{\lvert\delta^-_{ATM111}\rvert \le \lvert\delta^-_{MDM2201}\rvert + \delta^-_{ATM200} + \delta^+_{MDM2210}} 011 \xrightarrow{\delta^-_{P53011} \le \delta^-_{MDM2011}} \mathbf{001}$
5	$201 \xrightarrow{\delta^+_{P53201} \le \delta^-_{MDM2201} + \delta^-_{ATM201}} 211 \xrightarrow{\lvert\delta^-_{ATM211}\rvert \le \lvert\delta^-_{P53111}\rvert + 2\delta^+_{P53201} + \delta^-_{MDM2111}} 111 \xrightarrow{\lvert\delta^-_{ATM111}\rvert \le \lvert\delta^-_{MDM2201}\rvert + \delta^-_{ATM200} + \delta^+_{MDM2210}} 011 \xrightarrow{\delta^-_{MDM2011} \le \delta^-_{P53011}} \mathbf{010}$
6	$201 \xrightarrow{\delta^+_{P53201} \le \delta^-_{MDM2201} + \delta^-_{ATM201}} 211 \xrightarrow{\lvert\delta^-_{ATM211}\rvert \le \lvert\delta^-_{P53111}\rvert + 2\delta^+_{P53201} + \delta^-_{MDM2111}} 111 \xrightarrow{\delta^-_{P53111} \le + \delta^+_{P53201} + \delta^-_{ATM211}} 101 \xrightarrow{\delta^-_{ATM101} \le \delta^-_{MDM2101}} \mathbf{001}$
7	$201 \xrightarrow{\delta^+_{P53201} \le \delta^-_{MDM2201} + \delta^-_{ATM201}} 211 \xrightarrow{\lvert\delta^-_{ATM211}\rvert \le \lvert\delta^-_{P53111}\rvert + 2\delta^+_{P53201} + \delta^-_{MDM2111}} 111 \xrightarrow{\delta^-_{P53111} \le + \delta^+_{P53201} + \delta^-_{ATM211}} 101 \xrightarrow{\delta^-_{MDM2101} \le \delta^-_{ATM201}} 100$ $\xrightarrow{\lvert\delta^-_{ATM100}\rvert \le \delta^+_{P53201} + \delta^-_{MDM2101}} 000 \xrightarrow{\delta^+_{MDM2000} \le \delta^-_{P53000}} \mathbf{001}$
8	$201 \xrightarrow{\delta^+_{P53201} \le \delta^-_{MDM2201} + \delta^-_{ATM201}} 211 \xrightarrow{\lvert\delta^-_{ATM211}\rvert \le \lvert\delta^-_{P53111}\rvert + 2\delta^+_{P53201} + \delta^-_{MDM2111}} 111 \xrightarrow{\delta^-_{P53111} \le + \delta^+_{P53201} + \delta^-_{ATM211}} 101 \xrightarrow{\delta^-_{MDM2101} \le \delta^-_{ATM201}}$ $100 \xrightarrow{\lvert\delta^-_{ATM100}\rvert \le \delta^+_{P53201} + \delta^-_{MDM2101}} 000 \xrightarrow{\delta^+_{P53000} \le \delta^+_{MDM2000}} \mathbf{010}$
9	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert \le \lvert\delta^-_{ATM201}\rvert} 200 \xrightarrow{\delta^-_{ATM200} \le \delta^-_{MDM2201}} 100 \xrightarrow{\lvert\delta^-_{ATM100}\rvert \le \delta^+_{P53201} + \delta^-_{MDM2101}} 000 \xrightarrow{\delta^+_{MDM2000} \le \delta^-_{P53000}} \mathbf{001}$

10	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert \le \lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^-_{ATM200} \le \delta^-_{MDM2201}} 100 \xrightarrow{\lvert\delta^-_{ATM100}\rvert \le \delta^+_{P53201} + \delta^-_{MDM2101}} 000 \xrightarrow{\delta^+_{MDM2000} \le \delta^-_{P53000}} \mathbf{001}$
11	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert \le \lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^+_{P53200} \le \lvert\delta^-_{MDM201}\rvert} 210 \xrightarrow{\lvert\delta^-_{ATM200}\rvert \le \delta^+_{P53200}} 110 \xrightarrow{\delta^-_{ATM110} \le \delta^+_{MDM2111}} \mathbf{010}$
12	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert \le \lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^+_{P53200} \le \lvert\delta^-_{MDM201}\rvert} 210 \xrightarrow{\lvert\delta^-_{ATM200}\rvert \le \delta^+_{P53200}} 110 \xrightarrow{\delta^+_{MDM2210} \le \delta^-_{MDM2201} + \lvert\delta^-_{ATM210}\rvert} 111 \xrightarrow{\delta^-_{P53111} \le + \delta^+_{P53201} + \delta^-_{ATM211}} 101 \xrightarrow{\delta^-_{ATM101} \le \delta^-_{MDM2101}} \mathbf{001}$
13	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert \le \lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^+_{P53200} \le \lvert\delta^-_{MDM201}\rvert} 210 \xrightarrow{\lvert\delta^-_{ATM200}\rvert \le \delta^+_{P53200}} 110 \xrightarrow{\delta^+_{MDM2210} \le \delta^-_{MDM2201} + \lvert\delta^-_{ATM210}\rvert} 111 \xrightarrow{\delta^-_{P53111} \le + \delta^+_{P53201} + \delta^-_{ATM211}} 101 \xrightarrow{\delta^-_{MDM2101} \le \delta^-_{ATM201}} 100 \xrightarrow{\lvert\delta^-_{ATM100}\rvert \le \delta^+_{P53201} + \delta^-_{MDM2101}} 000 \xrightarrow{\delta^+_{MDM2000} \le \delta^-_{P53000}} \mathbf{001}$
14	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert \le \lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^+_{P53200} \le \lvert\delta^-_{MDM201}\rvert} 210 \xrightarrow{\lvert\delta^-_{ATM200}\rvert \le \delta^+_{P53200}} 110 \xrightarrow{\delta^+_{MDM2210} \le \delta^-_{MDM2201} + \lvert\delta^-_{ATM210}\rvert} 111 \xrightarrow{\delta^-_{P53111} \le + \delta^+_{P53201} + \delta^-_{ATM211}} 101 \xrightarrow{\delta^-_{MDM2101} \le \delta^-_{ATM201}} 100 \xrightarrow{\lvert\delta^-_{ATM100}\rvert \le \delta^+_{P53201} + \delta^-_{MDM2101}} 000 \xrightarrow{\delta^+_{P53000} \le \delta^+_{MDM2000}} \mathbf{010}$
15	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert \le \lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^+_{P53200} \le \lvert\delta^-_{MDM201}\rvert} 210 \xrightarrow{\lvert\delta^-_{ATM200}\rvert \le \delta^+_{P53200}} 110 \xrightarrow{\delta^-_{ATM110} \le \delta^+_{MDM2111}} \mathbf{010}$
16	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert \le \lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^+_{P53200} \le \lvert\delta^-_{MDM201}\rvert} 210 \xrightarrow{\lvert\delta^-_{ATM200}\rvert \le \delta^+_{P53200}} 110 \xrightarrow{\delta^+_{MDM2210} \le \delta^-_{MDM2201} + \lvert\delta^-_{ATM210}\rvert} 111 \xrightarrow{\delta^-_{P53111} \le + \delta^+_{P53201} + \delta^-_{ATM211}} 101 \xrightarrow{\delta^-_{ATM101} \le \delta^-_{MDM2101}} \mathbf{001}$
17	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert \le \lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^+_{P53200} \le \lvert\delta^-_{MDM201}\rvert} 210 \xrightarrow{\lvert\delta^-_{ATM200}\rvert \le \delta^+_{P53200}} 110 \xrightarrow{\delta^+_{MDM2210} \le \delta^-_{MDM2201} + \lvert\delta^-_{ATM210}\rvert} 111 \xrightarrow{\delta^-_{P53111} \le + \delta^+_{P53201} + \delta^-_{ATM211}} 101 \xrightarrow{\delta^-_{ATM101} \le \delta^-_{MDM2101}} \mathbf{001}$

18	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert\le\lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^+_{P53200}\le\lvert\delta^-_{MDM201}\rvert} 210 \xrightarrow{\lvert\delta^-_{ATM200}\rvert\le\delta^+_{P53200}} 110 \xrightarrow{\delta^+_{MDM2210}\le\delta^-_{MDM2201}+\lvert\delta^-_{ATM210}\rvert} 111 \xrightarrow{\delta^-_{P53111}\le\ +\ \delta^+_{P53201}+\delta^-_{ATM211}}$ $101 \xrightarrow{\delta^-_{MDM2101}\le\delta^-_{ATM201}} 100 \xrightarrow{\lvert\delta^-_{ATM100}\rvert\le\ \delta^+_{P53201}+\delta^-_{MDM2101}} 000 \xrightarrow{\delta^+_{MDM2000}\le\ \delta^-_{P53000}} \mathbf{001}$
19	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert\le\lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^+_{P53200}\le\lvert\delta^-_{MDM201}\rvert} 210 \xrightarrow{\lvert\delta^-_{ATM200}\rvert\le\delta^+_{P53200}} 110 \xrightarrow{\delta^+_{MDM2210}\le\delta^-_{MDM2201}+\lvert\delta^-_{ATM210}\rvert} 111 \xrightarrow{\delta^-_{P53111}\le\ +\ \delta^+_{P53201}+\delta^-_{ATM211}}$ $101 \xrightarrow{\delta^-_{MDM2101}\le\delta^-_{ATM201}} 100 \xrightarrow{\lvert\delta^-_{ATM100}\rvert\le\ \delta^+_{P53201}+\delta^-_{MDM2101}} 000 \xrightarrow{\delta^+_{P53000}\le\ \delta^+_{MDM2000}} \mathbf{010}$
20	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert\le\lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^+_{P53200}\le\lvert\delta^-_{MDM201}\rvert} 210 \xrightarrow{\delta^+_{P53201}\le\delta^-_{ATM201}} 211 \xrightarrow{\lvert\delta^-_{ATM211}\rvert\le\ \lvert\delta^-_{P53111}\rvert\ +2\delta^+_{P53201}+\delta^-_{MDM2111}} 111$ $\xrightarrow{\lvert\delta^-_{ATM111}\rvert\le\ \lvert\delta^-_{MDM2201}\rvert\ +\ \delta^-_{ATM200}+\delta^+_{MDM2210}} 011 \xrightarrow{\delta^-_{P53011}\le\ \delta^-_{MDM2011}} \mathbf{001}$
21	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert\le\lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^+_{P53200}\le\lvert\delta^-_{MDM201}\rvert} 210 \xrightarrow{\delta^+_{P53201}\le\delta^-_{ATM201}} 211 \xrightarrow{\lvert\delta^-_{ATM211}\rvert\le\ \lvert\delta^-_{P53111}\rvert\ +2\delta^+_{P53201}+\delta^-_{MDM2111}} 111$ $\xrightarrow{\lvert\delta^-_{ATM111}\rvert\le\ \lvert\delta^-_{MDM2201}\rvert\ +\ \delta^-_{ATM200}+\delta^+_{MDM2210}} 011 \xrightarrow{\delta^-_{MDM2011}\le\delta^-_{P53011}} \mathbf{010}$
22	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert\le\lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^+_{P53200}\le\lvert\delta^-_{MDM201}\rvert} 210 \xrightarrow{\delta^+_{P53201}\le\delta^-_{ATM201}} 211 \xrightarrow{\lvert\delta^-_{ATM211}\rvert\le\ \lvert\delta^-_{P53111}\rvert\ +2\delta^+_{P53201}+\delta^-_{MDM2111}} 111 \xrightarrow{\delta^-_{P53111}\le\ +\ \delta^+_{P53201}+\delta^-_{ATM211}}$ $101 \xrightarrow{\delta^-_{ATM101}\le\ \delta^-_{MDM2101}} \mathbf{001}$
23	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert\le\lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^+_{P53200}\le\lvert\delta^-_{MDM201}\rvert} 210 \xrightarrow{\delta^+_{P53201}\le\delta^-_{ATM201}} 211 \xrightarrow{\lvert\delta^-_{ATM211}\rvert\le\ \lvert\delta^-_{P53111}\rvert\ +2\delta^+_{P53201}+\delta^-_{MDM2111}} 111 \xrightarrow{\delta^-_{P53111}\le\ +\ \delta^+_{P53201}+\delta^-_{ATM211}}$ $101 \xrightarrow{\delta^-_{MDM2101}\le\delta^-_{ATM201}} 100 \xrightarrow{\lvert\delta^-_{ATM100}\rvert\le\ \delta^+_{P53201}+\delta^-_{MDM2101}} 000 \xrightarrow{\delta^+_{P53000}\le\ \delta^+_{MDM2000}} \mathbf{010}$
24	$201 \xrightarrow{\lvert\delta^-_{MDM2201}\rvert\le\lvert\delta^-_{ATM201}} 200 \xrightarrow{\delta^+_{P53200}\le\lvert\delta^-_{MDM201}\rvert} 210 \xrightarrow{\delta^+_{P53201}\le\delta^-_{ATM201}} 211 \xrightarrow{\lvert\delta^-_{ATM211}\rvert\le\ \lvert\delta^-_{P53111}\rvert\ +2\delta^+_{P53201}+\delta^-_{MDM2111}} 111 \xrightarrow{\delta^-_{P53111}\le\ +\ \delta^+_{P53201}+\delta^-_{ATM211}}$ $101 \xrightarrow{\delta^-_{MDM2101}\le\delta^-_{ATM201}} 100 \xrightarrow{\lvert\delta^-_{ATM100}\rvert\le\ \delta^+_{P53201}+\delta^-_{MDM2101}} 000 \xrightarrow{\delta^+_{MDM2000}\le\ \delta^-_{P53000}} \mathbf{001}$

The above trajectory shows that delay of ATM should be less than Mdm2 and next says δ^{-}_{ATM201} is less than delay of Mdm2 at location 201 and 101. Otherwise it might diverge from this pathway and can enter in any other pathways mentioned in table 3.7.

These delays are helpful in designing experiments on p53 in case of cancer as it is giving temporal delays for the states involved in a pathway moving towards stable steady state.

3.7. Invariance Kernel Results

The 2nd and 3rd Model has been used to find out the invariance kernels because of presence of cycle. Although same cycle is observed in both studies but the time delays for the same cycle in both studies are different. This different in time delays is due to different parameter optimization. The cycle used for the invariance kernel is given in figure 3.7.

3.7.1. Invariance Kernel Result for 2nd Model

The invariance kernels obtain for the cycle is given as follow. The constraints given below are generated by using the software **HYTech**. In the constraints given below dn represents the negative time delay same as δ^{-} and dp represents the positive time delay same as δ^{+}.

dnMDM2 <= dnP53 + dpMDM2 & dpP53 + 2dnMDM2 <= dnP53 + dpMDM2 & 2dnP53 + dpMDM2 <= dpP53 + dnMDM2 & 2dnP53 + dpMDM2 <= 2dpP53 + 2dnMDM2 & dnP53 + dpMDM2 <= 2dpP53 + dnMDM2 & dpMDM2 >= 0 & dpP53 + dnMDM2 <= dpMDM2 & dnP53 + dpMDM2 <= dpP53

After removing the non-trivial invariance kernels and by re-arranging following constraints were obtained:

1. dnMDM2 <= dnP53 + dpMDM2
2. 2dnP53 + dpMDM2 <= dpP53 + dnMDM2
3. 2dnP53 + dpMDM2 <= 2dpP53 + 2dnMDM2
4. dpP53 + 2dnMDM2 <= dnP53 + dpMDM2
5. 2dpP53 + 3dnMDM2 <= 2dnP53 + 2dpMDM2
6. dnP53 + dpMDM2 <= 2dpP53 + dnMDM2
7. dpP53 + dnMDM2 <= dpMDM2
8. dnP53 + dpMDM2 <= dpP53

Equation 1: dnMDM2 <= dnP53 + dpMDM2

It can be rearranged as:

$$\delta^{+}_{MDM2} + \delta^{-}_{MDM2} \geq |\delta^{-}_{P53}|$$

or

$$\pi\,(\text{Mdm2}) >= \text{dnP53}$$

π in the equation is showing the period as already discussed in section 2.5.

This equation shows that the time delay for both, the activation and inhibition of Mdm2is greater then inhibition period of p53. Graph in figure 3.9(a) gives more clear representation of this equation.

Equation 2: 2dnP53 + dpMDM2 <= dpP53 + dnMDM2

It can be rearranged as:

$$\delta^{+}_{MDM2} + |\delta^{-}_{MDM2}| \leq \delta^{+}_{P53} + |2\,\delta^{-}_{P53}|$$

or

$$(\text{Mdm2}) <= (\text{p53})$$

Equation 3: 2dnP53 + dpMDM2 <= 2dpP53 + 2dnMDM2

It can be rearranged as:

$$\delta^{+}_{MDM2} + |2\delta^{-}_{MDM2}| \leq 2\,\delta^{+}_{P53} + |2\,\delta^{-}_{P53}|$$

or

$$\pi\,(\text{Mdm2}) <= \pi\,(\text{p53})$$

Equation 4: dnP53 + dpMDM2 <= 2dpP53 + dnMDM2

It can be rearranged as:

$$\delta^{+}_{MDM2} + |\delta^{-}_{MDM2}| \leq 2\,\delta^{+}_{P53} + |\,\delta^{-}_{P53}|$$

or

$$\pi\,(\text{Mdm2}) <= (\text{p53})$$

Equation 2, 3 and 4 shows that delay period of Mdm2 is less than or equal to the delay period of p53. Delay constrains require for the activation and degradation of Mdm2 is less than that of required for the activation and inhibition of p53 as shown in figure 3.9 (b).

Equation 5: dpP53 + 2dnMDM2 <= dnP53 + dpMDM2

It can be rearranged as:

$$\delta^{+}_{P53} + |\,\delta^{-}_{P53}| \leq \delta^{+}_{MDM2} + |2\delta^{-}_{MDM2}|$$

or

$$\pi \text{ (p53)} <= \pi \text{ (Mdm2)}$$

Equation 6: 2dpP53 + 3dnMDM2 <= 2dnP53 + 2dpMDM2

It can be rearranged as:

$$2\delta^{+}_{P53} + \ |2\delta^{-}_{P53}| \leq 2\delta^{+}_{MDM2} + |3\delta^{-}_{MDM2}|$$

$$\pi \text{ (p53)} <= \pi \text{ (Mdm2)}$$

By looking at equation 5 and 6 it is clear that period of p53 is less than or equal to the period of Mdm2. Time period necessitate for p53 activity can be less than or equal to that of involve in activity of Mdm2. Graph in figure 3.9(c) best illustrate this relation.

Equation 7: dpP53 + dnMDM2 <= dpMDM2

It can be rearranged as:

$$\delta^{+}_{P53} \leq \delta^{+}_{MDM2} + |\delta^{-}_{MDM2}|$$

or

$$\text{dp p53} <= \pi(\text{Mdm2})$$

From this figure we can only analyze about the activation period of p53. Times require for the positive delay of p53 is less than the combine time for both the activation and inhibition of Mdm2 as given in figure 3.9(d)

Equation 8: dnP53 + dpMDM2 <= dpP53

It can be rearranged as:

$$\delta^{+}_{MDM2} \leq \delta^{+}_{P53} + |\delta^{-}_{P53}|$$

$$0r$$

$$\text{dp (Mdm2)} <= \pi(\text{P53})$$

This equation is giving clue only about the activation instance of Mdm2 at any iteration. Delay constrains for the positive delay of Mdm2 is less than the combine delay time of p53 activation and inhibition as shown in figure 3.10 (a).

After careful analyses of the equation generated for delay constrains, it is observed that the cycle is unstable and graph generated on the bases of these delays will show damping oscillations with decreasing peaks as apparent in figure 3.10(b). As cycle is unstable so iterations are limited to 3 and co-efficient up to level three are observed.

The co-efficient 1 mean that the time required for the activation or degradation of protein in one period is 5 minutes. In the next iteration if the co-efficient became 2, means that time has been increased, now that same protein will take 10 minutes for activation or degradation in next period or cycle and so on. The graph with the damping oscillation following the same behavior as mentioned in the equations is shown in fig 3.10 (b).

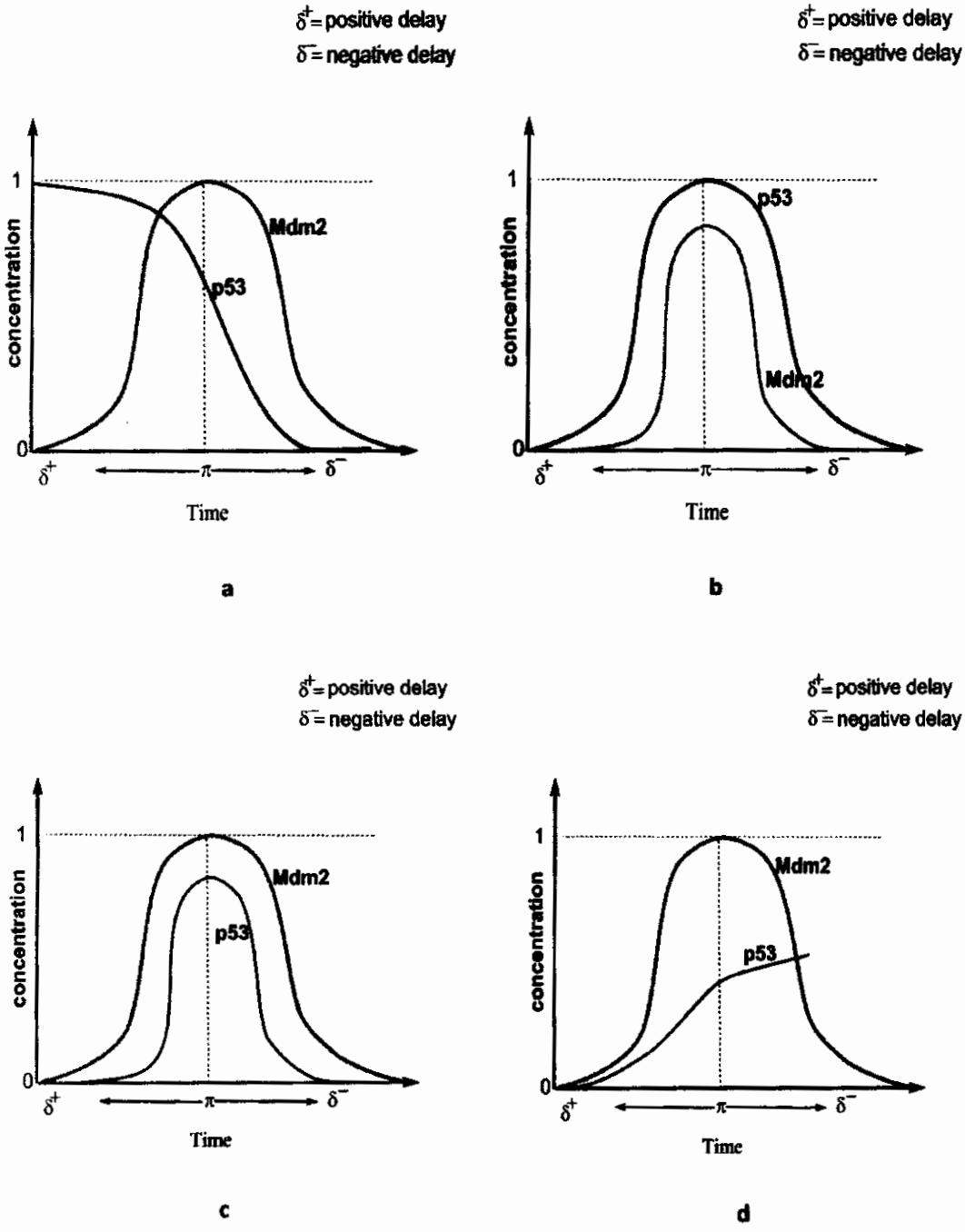


Figure 3.9: (a) Graphical representation of equation 1 **(b)** Graphical representation of equation 2, 3 and 4 **(c)** Graphical representation of e equation 5 and 6 **(d)** Graphical representation of equation 7.

3.7.2 Invariance Kernel Result for 3rd Model

The invariance kernels obtain for the cycle in 3rd Model by using **HYTech** is given as follow.

dnMDM2 = 0 & dpP53 = dnP53 + dpMDM2 & dnP53 <= 0 &dnP53 + dpMDM2>= 0

After removing the non-trivial invariance kernels and by re-arranging following constraints were obtained:

8. dnP53 + dpMDM2 >= 0

9. dpP53 = dnP53 + dpMDM2

After removing non trivial kernels two important constrains are left which are explained as below.

Equation 8: dnP53 + dpMDM2 >= 0

It can be rearranged as:

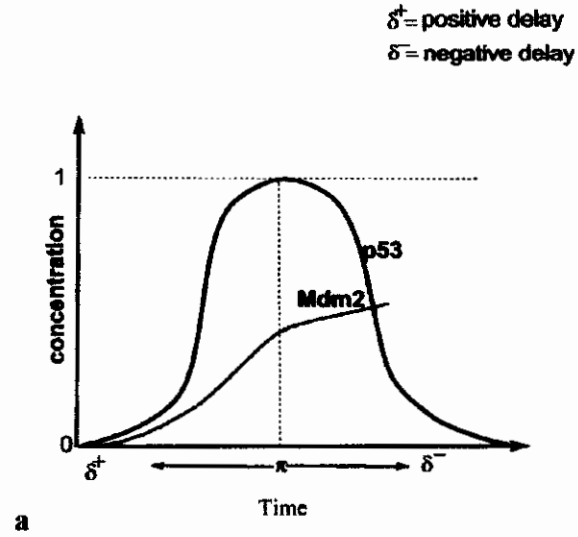
$$\left| \delta_{p53}^{-} \right| \leq \delta_{MDM2}^{+}$$

This equation shows that the inhibition or degradation of p53 must be less than or equal to the positive time delay of Mdm2. Graphical representation of the equation can be seen in figure 3.11 (a).

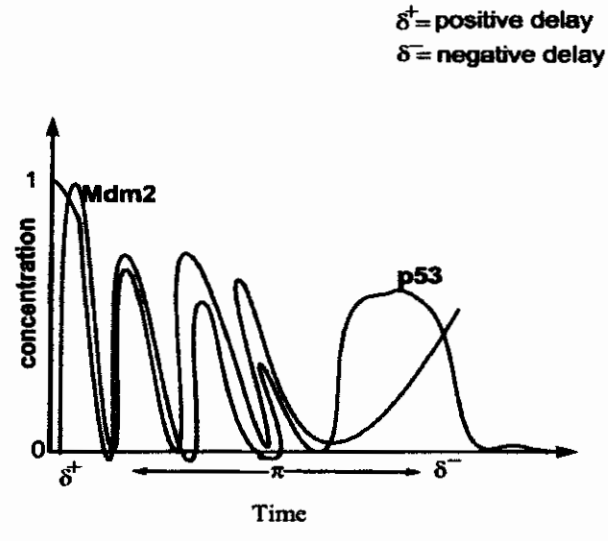
Equation 9: dpP53 = dnP53 + dpMDM2

It can be rearranged as:

$$\delta_{p53}^{+} + \left| \delta_{p53}^{-} \right| = \delta_{MDM2}^{+}$$



a



b

Figure 3.10: (a) Graphical representation of equation 8 **(b)** Damping oscillation showing the result of all equations

or

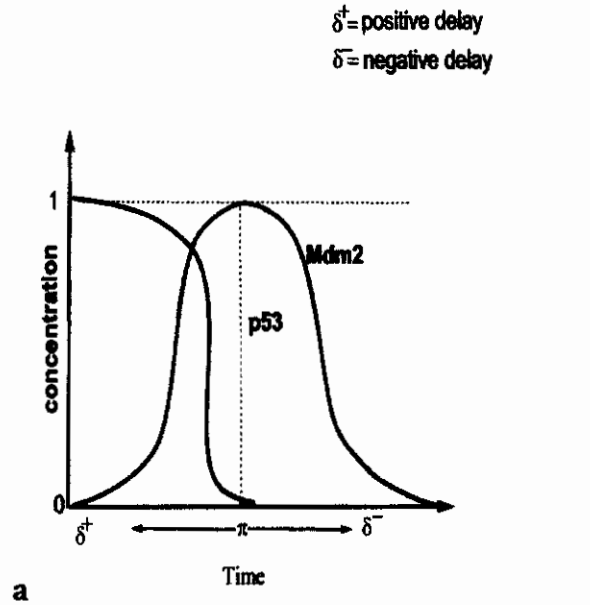
$$\pi\,(\mathrm{p53}) = \delta^{+}_{MDM2101}$$

The above equation represents very important constrain of our study. This equation depicts that in order to maintain homeostasis in the system, both positive and negative delay of p53 is equal to the positive delay of Mdm2. It can be further explained as that the time required for the concentration built up of Mdm2 is exactly equal to the time require for both the activation (concentration build up) and degradation of p53. It means that the activity of p53 is faster as compare to the activity of Mdm2 with in cycle. This type of behavior is given in graphical form in figure 3.11(b).

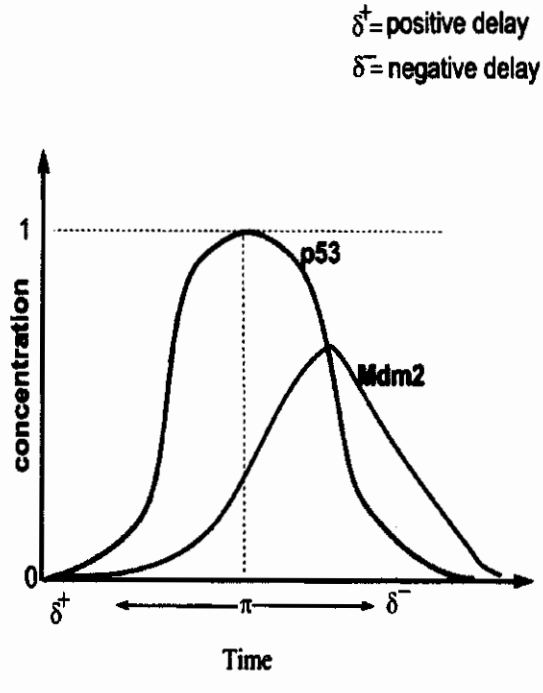
Both the models used for finding the invariance kernel are showing different results. 2nd model is representing that it has unstable cycle. As there is no equality sign in constraints generated, is an indication of instability in the behavior of the system. The graph of unstable cycle shows damp oscillation with decreasing peaks as shown in figure 3.12(a). Depending on the parameter values, a damp oscillation can produce graph with altering degree of damping, amplitude and oscillation period.

3rd Model shows the clue of stable cycle. The constrained obtained from 3rd model has equality sign in it. The presence of equality is an indication of stable behavior of the system. The graph of unstable cycle shows expanding oscillation or sustained oscillation shown figure3.12 (b). The type of oscillations given in figure 3.12(b) are known as sustained oscillation because oscillations in each cycle is identical to one in the previous, the maximum concentration of protein (in case of BRNs), known as peak is same in each cycle and oscillation periods (time taken to complete one cycle) are of same length. The phenomena of stable and unstable behavior of the cycle can

best illustrated by the diagram shown in figure 3.13. Figure 3.13 (left) depicts the unstable behavior of the cycle and figure 3.13 (right) explain the stable behavior of cycle in the system.



a



b

Figure 3.11: (a) Graphical representation of equation 9 **(b)** Graphical representation of equation 10

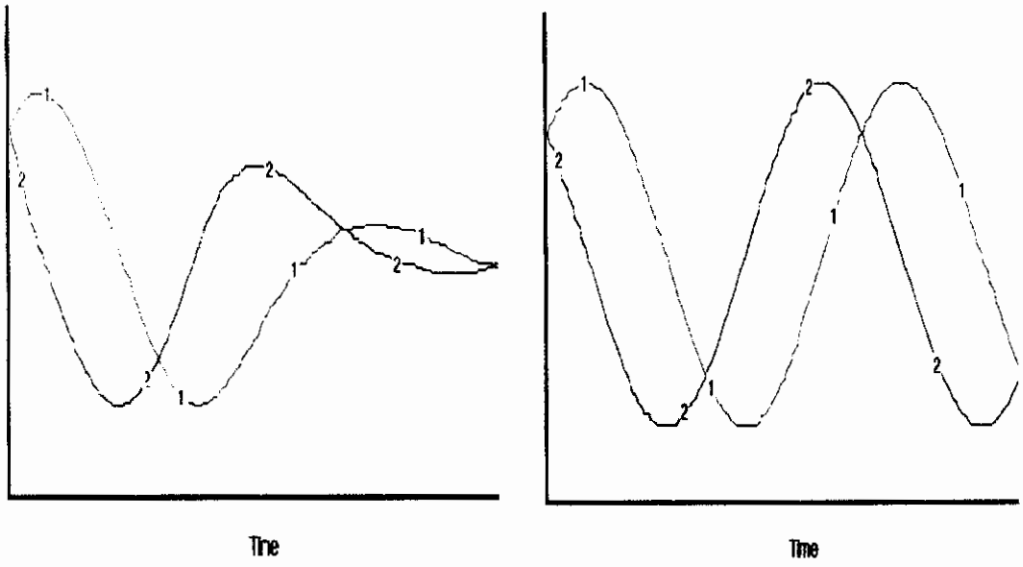


Figure 3.12: (a) Example of damp oscillation **(b)** Example of sustained oscillation

(Kamin *et al.*, 2002)

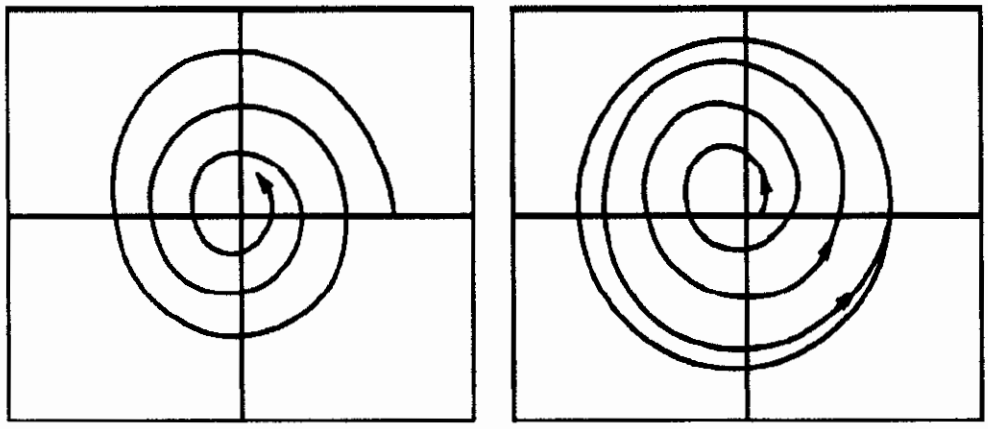


Figure 3.13: unstable cyclic behavior (left) and stable cyclic behavior (right)

(Ahmed *et al.*, 2009)

CHAPTER 4

DISCUSSION

4. DISCUSSION

In the case of p53 system, the function of p53 is to transcriptionally activate Mdm2. In turn, p53 is negatively regulated by Mdm2. Mdm2 normalize p53 at two distinct ways, by enhancing its degradation rate and as a transcription factor by inhibiting its activity thus making negative feedback loop (Momand *et al*, 2000; Haupt *et al*, 1997; Piette *et al*, 1997). When there is a stress placed on the cell these interactions are modified so that p53 levels can increase and activate the necessary pathways (Balint and Vousden, 2001), the members of the p53 regulatory network interact and elicit modifications to the proteins involved in the network. These protein modifications modify the p53 protein by increasing its half life, from 6-20 minutes to an hour (Harris and Levine, 2005; Hickman *et al.*, 2002). This process is very fast, p53 levels increase within a minute and the first apoptotic events occur within a few hours in some cell types. These protein modifications also increasing the ability of the p53 to bind to specific DNA sequence and help in promoting the transcription of genes regulated by those DNA sequences (Overholtzer *et al.*, 2003).

Both the stable steady states i.e. 0, 0, 1 and 0, 1, 0 in 1st and 3rd model and 0, 0, 1 in 2nd model are in accordance with the fact that p53 is kept at low levels in unstressed cells due to MDM2-mediated degradation(Toledo and Wahl, 2007). One of our stable steady state is showing the same results i.e. 0,0,1 in which first 0 is representing the stress (ATM) which is absent and the 1 at third position is for mdm2 representing that mdm2 is present and thus keeps the p53 at low level. 2nd stable steady state is representing the abnormal behavior or diseased condition of the system i.e. 0, 1, 0 p53 (at second position) is 1 showing the over expression of p53. Over expression of p53 is reported in wide variety of cancer, the results of study in 2006 has confirm the

involvement of p53 in the growth regulation of bladder cancer (Lipponen *et al.*, 2006). Over expression of p53 is also found in breast carcinoma which is associated with point mutations found in highly conserved regions of the p53 gene (Davidoff *et al.*, 1991).

The objective of the research is to help in the cure of cancer at each state. This is accomplished by a state graph in Figure 3.2, 3.4 and 3.6. As it show all the possible states that can be generated in the BRN and it shows how a tumor progresses in the body, it shows all types and all possible trajectories followed by a tumor. This will help understanding the cancer progression i n an elaborative manner and help in clinical trials as each type and stage of patients can be diagnosed and treated. Like state transitions in Figure 3.6, the state table is also contributive as it provide information about each steady state i.e. how it changes to new state and which element is controlled by which element and in which states it can transit. It is a competent tool and it will be efficacious in controlling disease states and aid in moulding the states towards desirable states.

In our study we present simple qualitative model of p53-mdm2 feedback loop and tried to capture the gross mechanism of this feedback loop. We investigate that how numerically different parameters can affect the type of behavior that the system can exhibit. In the Boolean model (1^{st} model) cycle is not observed suggesting that stress signal is weak and ATM should be strongly phosphorylated by the strong signal in order to respond the stress. It is also confirming the study of Banin that enhanced activity of ATM is necessary in order to maintain homeostasis to respond the stress (Banin *et al.*,2000) otherwise system will lead to either of stable steady state.

It can also be interpreted as stress signal is not strong enough to maintain the homeostasis in the system. This is because stress will either subsides after single pulse generation of p53 that will be enough to overcome the stress or might be there is some mutation in p53 and system will lead to stable steady states depending on the respective situation.

When sufficiently strong damage signal is introduced to the model (multi valued model i.e. 2^{nd} and 3^{rd} model) it leads to the oscillatory behavior of both mdm2 and p53. In cycle when stress is triggered it decrease the degradation of p53 by increasing the degradation of Mdm2.This process help p53 to rise above its basal level and in the transcription of other downstream genes along with its transcription factor mdm2. The activation of downstream genes will help in responding stress by arresting cell cycle, apoptosis e.t.c and activation of Mdm2 will decrease the level of p53 again. If still there is enough damage or stress the whole process or cycle will persist until stress subsides. These results are very much similar to the study of Ruth Lev Bar-Or and colleagues, they have predicted the same results using mathematical modeling (Ruth *et al.*, 2000).

Hybrid Modeling generate time delay feature as shown in Table 3.7 for steady stable steady states trajectories and homeostasis. It will help maintaining system in specified trajectory by altering these delays and lengthening of the specific constraints to keep the system in desirable steady state. It provides us the pathway followed by trajectories along each dimension and these delays are Hybrid constraints of dynamical system. The Table 3.8, is labeling whole pathways, derived from state graph Figure 3.6, can be used further for designing controlled medium for tumor

control and providing guidelines for clinical experiments designing. Although cycles generated in both 2nd and 3rd model by the qualitative modeling is same but after applying hybrid modeling they are exhibiting different behavior due to different parameter optimization. As observed from constraints given in section 3.7, oscillations of two different types are evident i.e. damped oscillation and sustained or perfect oscillation.

2nd model is exhibiting damped oscillations as clearly shown in graph of figure 3.10 (b).3rd model is exhibiting the sustained oscillation, as clue from graph in figure 3.11(a) and (b). If we combine this type of graph (given in figure 3.11(a) and (b)) up to 3 or 4 oscillations then the resulting graph will be the same as shown in figure 4.1 from the study of Carole J proctor and Douglas A Gray (proctor and Gray, 2008).

All the results from our study are very much in accordance the natural phenomena of the body that has been already experimentally observed in wet lab and has been predicted by using mathematical modeling (Hunziker *et al.*, 2010; proctor and Gray, 2008; zatorsky *et al.*,2006; Ruth *et al.*, 2000). In some papers any combination of low or high level of Mdm2 and p53 is evident in stable steady states same as of our results (Hunziker *et al.*, 2010). Likewise our results oscillations are generated in all most all studies (Hunziker *et al.*, 2010; proctor and Gray, 2008; zatorsky *et al.*, 2006; Ruth *et al.*, 2000). These oscillations could be taken as repair efforts for the damage e.g in an oscillation a pulse of p53 generates and start the transcription of other down steam genes which elicit cell cycle arrest, apoptosis and DNA repair in order to respond the stress and fix it properly. If stress or damage persists after the generation of first pulse another pulse will be generated and so on until the stress is resolved and the stress

signal that had activated the p53 subsides. Duration of p53 peaks in oscillation depends on severity of the damage as reported in our study (Ahmed *et al.*, 2011).

The analysis of whole study indicates the homeostasis (cycle) is a control mechanism. Pluses have been generated due to increased level of p53 in oscillations. As a result of these pluses downstream genes are transcribed for the fighting against the stress. If this control mechanism is disturbed it will lead to stable steady states.

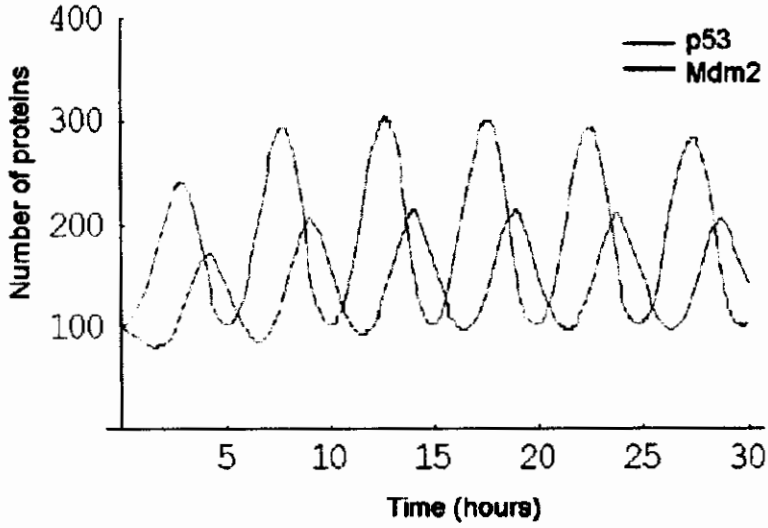


Figure 4.1: Graph showing oscillation between p53 and mdm2

(proctor and Gray, 2000)

4.1. Conclusion

The p53 associated GRNs are important tools for providing the best and possible drug designing target as the modeling of the BRN provide a deep insight into the behavior of the system, providing all trajectories towards homeostasis and deadlock states their threshold values for each state transition. It is further embellished by the introduction of time delay constraints in the BRN; they are providing the checkpoints for controlling protein activation and inhibition with respect to time context. The modeling formalism provide natural cancer controlling therapeutic targets as the BRN include all proteins regulating each other rather dealing each protein as a single target for inhibition and designing single inhibitors for each and every protein the qualitative and Hybrid modeling and analysis are dealing with whole phenomenon as a single entity.

4.2. Future Prospects

The GRN of p53 could be eventually extended to cover other entities that play vital role in triggering the p53 gene. By adding more entities in the models discussed above, exact therapeutic targets will be found by docking and simulation studies of the interacting proteins. These findings lead us towards drug designing. On the basis of modeling techniques, a computer aided tool for rapid analysis can also be developed. Real time modeling and its applications can help in predicting the preventive medicines, which will give the advancement in medicine developed for the treatment of cancer.

CHAPTER 5

REFERENCES

5. REFERENCES

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ANNEXURE

```
--- Fichier latest hytech file
var
hATM,hP53,hMDM2 :analog;
k,n: discrete;
dpATM, dnATM,dpP53,dnP53,dpMDM2, dnMDM2, dpP53000,
dpMDM2000,dnP53010,
 dpMDM2010,dnP53011,dnMDM2011,dnATM100, dpP53100, dpMDM2100,
dnATM101,
dpP53101,dnMDM2101,dnATM110, dnP53110, dpMDM2110, dnATM111,
dnP53111,
dnMDM2111, dnATM200, dpP53200,dnATM201,dpP53201,
dnMDM2201,dnATM210,
dnP53210, dpMDM2210,dnATM211, dnP53211, dnMDM2211, dpMDM2200,
dpMDM2101
:parameter;
automaton auto
synclabs: ;
-- gène n°0 = ATM
-- gène n°1 = P53
-- gène n°2 = MDM2
initially loc_201;

-- pour la configuration 0,0,0
loc loc_000: while hP53 <= dpP53000 & hMDM2 <= dpMDM2000 wait
{dhATM=0,dhP53=1,dhMDM2=1}
when hP53 = dpP53000 do {hP53' = 0, k'=k+1} goto loc_010;
when hMDM2 = dpMDM2000 do {hMDM2' = 0, k'=k+1} goto loc_001;

-- pour la configuration 0,0,1
loc loc_001: while True wait {dhATM=0,dhP53=0,dhMDM2=0}

-- pour la configuration 0,1,0
loc loc_010: while True wait {dhATM=0,dhP53=0,dhMDM2=0}

-- pour la configuration 0,1,1
loc loc_011: while hP53>= dnP53011 & hMDM2>= dnMDM2011  wait
{dhATM=0,dhP53=-1,dhMDM2=-1}
when hP53 = dnP53011 do {hP53' = 0, k'=k+1} goto loc_001;
when hMDM2 = dnMDM2011 do {hMDM2' = 0, k'=k+1} goto loc_010;
-- ok
-- pour la configuration 1,0,0
loc loc_100: while hATM >= dnATM100 & hP53 <= dpP53100 &
hMDM2 <= dpMDM2100 wait {dhATM=-1,dhP53=1,dhMDM2=1}
when hATM = dnATM100 do { hATM'= 0, k'=k+1} goto loc_000;
when hP53 = dpP53100 do {hP53'= 0, k'=k+1} goto loc_110;
--when hMDM2 = dpMDM2100 do {hMDM2'=0, k'=k+1} goto loc_101;
--ok
-- pour la configuration 1,0,1
```

```
loc loc_101: while hATM >= dnATM101 & hP53 <= dpP53101 & hMDM2
>= dnMDM2101 wait {dhATM=-1,dhP53=1,dhMDM2=-1}
when hATM = dnATM101 do {hATM'= 0, k'=k+1} goto loc_001;
--when  hP53 = dpP53101  do {hP53'= 0, k'=k+1} goto loc_111;
when hMDM2 = dnMDM2101 do {hMDM2'=0, k'=k+1} goto loc_100;
--ok
-- pour la configuration 1,1,0
loc loc_110: while hATM >= dnATM110 & hP53 >= dnP53110 & hMDM2
<= dpMDM2110 wait {dhATM=-1,dhP53=-1,dhMDM2=1}
when hATM = dnATM110 do { hATM'= 0, k'=k+1} goto loc_010;
--when hP53 = dnP53110 do {hP53'= 0, k'=k+1} goto loc_100;
when hMDM2 = dpMDM2110 do {hMDM2'=0, k'=k+1} goto loc_111;
--ok
-- pour la configuration 1,1,1
loc loc_111: while hATM >= dnATM111 & hP53 >= dnP53111 & hMDM2
>= dnMDM2111 wait {dhATM=-1,dhP53=-1,dhMDM2=-1}
when hATM = dnATM111 do { hATM'= 0, k'=k+1} goto loc_011;
when hP53 = dnP53111 do {hP53'= 0, k'=k+1} goto loc_101;
--when hMDM2 = dnMDM2111 do {hMDM2'=0, k'=k+1} goto loc_110;

-- pour la configuration 2,0,0
loc loc_200: while hATM >= dnATM200 & hP53 <= dpP53200 & hMDM2
<= dpMDM2200  wait {dhATM=-1,dhP53=1,dhMDM2=1}
when hATM = dnATM200 do { hATM'= 0, k'=k+1} goto loc_100;
when hP53 = dpP53200 do {hP53'= 0, k'=k+1} goto loc_210;
--when hMDM2 = dpMDM2200 do {hMDM2'= 0, k'=k+1} goto loc_201;

-- pour la configuration 2,0,1
loc loc_201: while hATM >= dnATM201 & hP53 <= dpP53201 & hMDM2
>=dnMDM2201 wait {dhATM=-1,dhP53=1,dhMDM2=-1}
when hATM = dnATM201 do { hATM'= 0, k'=k+1} goto loc_101;
when hP53 = dpP53201 do {hP53'= 0, k'=k+1} goto loc_211;
when hMDM2 = dnMDM2201 do {hMDM2'=0, k'=k+1} goto loc_200;

-- pour la configuration 2,1,0
loc loc_210: while hATM >= dnATM210 & hP53 >= dnP53210 & hMDM2
<= dpMDM2210 wait {dhATM=-1,dhP53=0,dhMDM2=1}
when hATM = dnATM210 do { hATM'= 0, k'=k+1} goto loc_110;
--when hMDM2 = dpMDM2210 do {hMDM2'=0, k'=k+1} goto loc_211;

-- pour la configuration 2,1,1
loc loc_211: while hATM >= dnATM211 & hP53 >= dnP53211 & hMDM2
>= dnMDM2211 wait {dhATM=-1,dhP53=-1,dhMDM2=0}
when hATM = dnATM211 do { hATM'= 0, k'=k+1} goto loc_111;
--when hP53 = dnP53211 do {hP53'= 0, k'=k+1}  goto loc_201;

end
var
ini_reg, access: region;

--initial region
```

```
ini_reg:= loc[auto] = loc_201 & hATM=0 & hP53=0 & hMDM2=0;

--/access all reachable states
access:=post(post(post(post(post(post(post(ini_reg)))))));
prints "=================================================";
prints "Accessible states from the initial state along with
the
parameter constraints";
print hide non_parameters in access endhide;
prints "=================================================";

-- Fichier hytechfile


var
hATM,hP53,hMDM2 :analog;
k,n: discrete;
dpATM2,dpATM, dnATM2, dnATM,dpP53,dnP53,dpMDM2, dnMDM2
 :parameter;
automaton auto
synclabs: ;
-- gène n°0 = ATM
-- gène n°1 = P53
-- gène n°2 = MDM2

initially loc_100;

-- pour la configuration 0,0,0
loc loc_000: while hP53 <= dpP53 & hMDM2 <= dpMDM2  wait
{dhATM=0,dhP53=1,dhMDM2=1}
when hP53 = dpP53 do {hP53' = 0, k'=k+1} goto loc_010;
when hMDM2 = dpMDM2 do {hMDM2' = 0, k'=k+1} goto loc_001;

-- pour la configuration 0,0,1
loc loc_001: while True wait {dhATM=0,dhP53=0,dhMDM2=0}

-- pour la configuration 0,1,0
loc loc_010: while hP53 >= dnP53 & hMDM2 <= dpMDM2 wait
{dhATM=0,dhP53=-1,dhMDM2=1}
when hP53 = dnP53 do {hP53'=0, k'=k+1} goto loc_000;
when hMDM2 = dpMDM2 do {hMDM2'=0, k'=k+1}  goto loc_011;

-- pour la configuration 0,1,1
loc loc_011: while hP53 >= dnP53  wait
{dhATM=0,dhP53=-1,dhMDM2=0}
when hP53 = dnP53 do {hP53'= 0, k'=k+1} goto loc_001;

-- pour la configuration 1,0,0
loc loc_100: while hATM >= dnATM & hP53 <= dpP53 &  hMDM2 <=
dpMDM2 wait
{dhATM=-1,dhP53=1,dhMDM2=1}
when  hATM = dnATM do { hATM'= 0, k'=k+1} goto loc_000;
```

```
when hP53 = dpP53 do {hP53'= 0, k'=k+1} goto loc_110;
----when hMDM2 = dpMDM2100 do {hMDM2'=0, k'=k+1} goto loc_101;

-- pour la configuration 1,0,1
loc loc_101: while hATM >= dnATM & hP53 <= dpP53 & hMDM2 >=
dnMDM2 wait
{dhATM=-1,dhP53=1,dhMDM2=-1}
when hATM = dnATM do {hATM'= 0, k'=k+1} goto loc_001;
--when hP53 = dpP53101  do {hP53'= 0, k'=k+1} goto loc_111;
when hMDM2 = dnMDM2 do {hMDM2'=0, k'=k+1} goto loc_100;

-- pour la configuration 1,1,0
loc loc_110: while hATM >= dnATM & hP53 >= dnP53 & hMDM2 <=
dpMDM2 wait
{dhATM=-1,dhP53=-1,dhMDM2=1}
when hATM = dnATM do { hATM'= 0, k'=k+1} goto loc_010;
--when hP53 = dnP53110 do {hP53'= 0, k'=k+1} goto loc_100;
when hMDM2 = dpMDM2 do {hMDM2'=0, k'=k+1} goto loc_111;

-- pour la configuration 1,1,1
loc loc_111: while hATM >= dnATM & hP53 >= dnP53 & hMDM2 >=
dnMDM2 wait
{dhATM=-1,dhP53=-1,dhMDM2=-1}
when hATM = dnATM do { hATM'= 0, k'=k+1} goto loc_011;
when hP53 = dnP53 do {hP53'= 0, k'=k+1} goto loc_101;
--when hMDM2 = dnMDM2111 do {hMDM2'=0, k'=k+1} goto loc_110;

-- pour la configuration 2,0,0
loc loc_200: while  hATM >= dnATM & hP53 <= dpP53 wait
{dhATM=-1,dhP53=1,dhMDM2=0}
when hATM = dnATM do { hATM'= 0, k'=k+1} goto loc_100;
--when hP53 = dpP53200 do {hP53'= 0, k'=k+1} goto loc_210;

-- pour la configuration 2,0,1
loc loc_201: while hATM >= dnATM & hP53 <= dpP53 & hMDM2
>=dnMDM2 wait
{dhATM=-1,dhP53=1,dhMDM2=-1}
when hATM = dnATM do { hATM'= 0, k'=k+1} goto loc_101;
when hP53 = dpP53 do {hP53'= 0, k'=k+1} goto loc_211;
when hMDM2 = dnMDM2 do {hMDM2'=0, k'=k+1} goto loc_200;

-- pour la configuration 2,1,0
loc loc_210: while hATM >= dnATM & hP53 >= dnP53 & hMDM2 <=
dpMDM2 wait
{dhATM=-1,dhP53=0,dhMDM2=1}
when hATM = dnATM do { hATM'= 0, k'=k+1} goto loc_110;
when hMDM2 = dpMDM2 do {hMDM2'=0, k'=k+1} goto loc_211;

-- pour la configuration 2,1,1
loc loc_211: while  hATM >= dnATM & hP53 >= dnP53 & hMDM2 >=
dnMDM2 wait
 {dhATM=-1,dhP53=-1,dhMDM2=0}
```

```
when hATM = dnATM2 do { hATM'= 0, k'=k+1} goto loc_111;
--when hP53 = dnP53211 do {hP53'= 0, k'=k+1}  goto loc_201;

end
var
init_reg, acces,
portrait,fstate,nes_cyc_length,pln_cyc_length,fixpoint,r_ini,r
_old,r_new,r_acc: region;

--var
  -- init_reg, acces : region;
init_reg := loc[auto] = loc_100 & hATM<=0 & hP53>=0  &
hMDM2>=0 & hATM >= dnATM & hP53 <= dpP53 &  hMDM2 <= dpMDM2;

-- -------------------Les variables--------------------------
acces:= hide k, n in post(post(post(post(init_reg & k=n) &
~k=n))) endhide;
init_reg:=hull(acces) & init_reg;

print hide hATM,hP53,hMDM2  in init_reg endhide;
acces:= hide k, n in post(post(post(post(init_reg & k=n) &
~k=n))) endhide;
init_reg:=hull(acces) & init_reg;
print hide hATM,hP53,hMDM2  in init_reg endhide;

acces:= hide k, n in post(post(post(post(init_reg & k=n) &
~k=n))) endhide;
init_reg:=hull(acces) & init_reg;
print hide hATM,hP53,hMDM2  in init_reg endhide;

-- Analysis commands
r_ini:= loc[auto] = loc_100 &  hP53>=0  &   hP53 <= dpP53;
--r_ini:= loc[auto] = loc_100 & hMDM2>=0 & hMDM2 <= dpMDM2100;
-- hP53>=0  & hMDM2>=0 &  hP53 <= dpP53100 &  hMDM2 <=
dpMDM2100;
r_new:=hide k,n in hull (post(r_ini & k=n) & ~k=n) endhide;
r_old:=r_ini & ~r_ini;

while not empty(r_new) and empty(r_new & r_ini) do
r_old:=r_new;
r_new:=hide k,n in hull(post(r_new & k=n) & ~k=n) endhide;
endwhile;
-- To verify that the initial zone is accessible from itself
if not empty (r_new & r_ini) then
-- if accessible
r_acc:=hide k,n in hull(post(r_new & k=n) &~k=n) endhide;
r_old:=r_ini & ~r_ini; --empty region initialization
while not empty(r_acc) and not r_new<=r_old do
r_old:=r_new;
while not empty(r_acc) and empty(r_acc & r_ini) do
r_acc:= hide k,n in hull(post(r_acc & k=n) &~k=n) endhide;
endwhile;
```

```
r_acc:=hull(r_acc & r_ini);
r_new:=hull(r_acc & r_new);
r_acc:=hide k,n in hull(post(r_new & k=n) & ~k=n) endhide;
endwhile;
if not empty(r_new) then
prints
"============================================================"
;
prints "Constrained region of the Invariance Kernel in the
zone:";
--print hide h in r_new endhide;
prints
"============================================================"
;
prints
"============================================================"
;
prints "Delay constraintes:";
print  hide hATM,hP53,hMDM2 in r_new endhide;
prints
"============================================================"
;
else
prints "Invariance kernel does not exist from the initial
region";
endif;
else
-- if not accessible
prints "The initial region is not accessible from itself
hence";
prints "there is no initial condition that leads to an
invariance kernel.";
endif;
-- Fichier latest hytech file-----
var
hATM,hP53,hMDM2 :analog;
k,n: discrete;
dpATM2,dpATM, dnATM2, dnATM,dpP53,dnP53,dpMDM2, dnMDM2
:parameter;
automaton auto
synclabs: ;
-- gène n°0 = ATM
-- gène n°1 = P53
-- gène n°2 = MDM2
initially loc_101;

-- pour la configuration 0,0,0
loc loc_000: while hP53 <= dpP53 & hMDM2 <= dpMDM2 wait
{dhATM=0,dhP53=1,dhMDM2=1}
when hP53 = dpP53 do {hP53' = 0, k'=k+1} goto loc_010;
when hMDM2 = dpMDM2 do {hMDM2' = 0, k'=k+1} goto loc_001;
```

```
-- pour la configuration 0,0,1
loc loc_001: while True wait {dhATM=0,dhP53=0,dhMDM2=0}

-- pour la configuration 0,1,0
loc loc_010: while True wait {dhATM=0,dhP53=0,dhMDM2=0}

-- pour la configuration 0,1,1
loc loc_011: while hP53>= dnP53 & hMDM2>= dnMDM2  wait
{dhATM=0,dhP53=-1,dhMDM2=-1}
when hP53 = dnP53 do {hP53' = 0, k'=k+1} goto loc_001;
when hMDM2 = dnMDM2 do {hMDM2' = 0, k'=k+1} goto loc_010;
-- ok
-- pour la configuration 1,0,0
loc loc_100: while hATM >= dnATM & hP53 <= dpP53 &  hMDM2 <=
dpMDM2 wait {dhATM=-1,dhP53=1,dhMDM2=1}
when hATM = dnATM do { hATM'= 0, k'=k+1} goto loc_000;
when hP53 = dpP53 do {hP53'= 0, k'=k+1} goto loc_110;
--when hMDM2 = dpMDM2100 do {hMDM2'=0, k'=k+1} goto loc_101;
--ok
-- pour la configuration 1,0,1
loc loc_101: while hATM >= dnATM & hP53 <= dpP53 & hMDM2 >=
dnMDM2 wait {dhATM=-1,dhP53=1,dhMDM2=-1}
when hATM = dnATM do {hATM'= 0, k'=k+1} goto loc_001;
--when  hP53 = dpP53101  do {hP53'= 0, k'=k+1} goto loc_111;
when hMDM2 = dnMDM2 do {hMDM2'=0, k'=k+1} goto loc_100;
--ok
-- pour la configuration 1,1,0
loc loc_110: while hATM >= dnATM & hP53 >= dnP53 & hMDM2 <=
dpMDM2 wait {dhATM=-1,dhP53=-1,dhMDM2=1}
when hATM = dnATM do { hATM'= 0, k'=k+1} goto loc_010;
--when hP53 = dnP53110 do {hP53'= 0, k'=k+1} goto loc_100;
when hMDM2 = dpMDM2 do {hMDM2'=0, k'=k+1} goto loc_111;
--ok
-- pour la configuration 1,1,1
loc loc_111: while hATM >= dnATM & hP53 >= dnP53 & hMDM2 >=
dnMDM2 wait {dhATM=-1,dhP53=-1,dhMDM2=-1}
when hATM = dnATM do { hATM'= 0, k'=k+1} goto loc_011;
when hP53 = dnP53 do {hP53'= 0, k'=k+1} goto loc_101;
--when hMDM2 = dnMDM2111 do {hMDM2'=0, k'=k+1} goto loc_110;

-- pour la configuration 2,0,0
loc loc_200: while hATM >= dnATM2 & hP53 <= dpP53 & hMDM2 <=
dpMDM2  wait {dhATM=-1,dhP53=1,dhMDM2=1}
when hATM = dnATM2 do { hATM'= 0, k'=k+1} goto loc_100;
when hP53 = dpP53 do {hP53'= 0, k'=k+1} goto loc_210;
--when hMDM2 = dpMDM2200 do {hMDM2'= 0, k'=k+1} goto loc_201;

-- pour la configuration 2,0,1
loc loc_201: while hATM >= dnATM2 & hP53 <= dpP53 & hMDM2
>=dnMDM2 wait {dhATM=-1,dhP53=1,dhMDM2=-1}
when hATM = dnATM2 do { hATM'= 0, k'=k+1} goto loc_101;
when hP53 = dpP53 do {hP53'= 0, k'=k+1} goto loc_211;
```

```
when hMDM2 = dnMDM2 do {hMDM2'=0, k'=k+1} goto loc_200;

-- pour la configuration 2,1,0
loc loc_210: while hATM >= dnATM2 & hP53 >= dnP53 & hMDM2 <=
dpMDM2 wait {dhATM=-1,dhP53=0,dhMDM2=1}
when hATM = dnATM2 do { hATM'= 0, k'=k+1} goto loc_110;
--when hMDM2 = dpMDM2210 do {hMDM2'=0, k'=k+1} goto loc_211;
-- pour la configuration 2,1,1
loc loc_211: while hATM >= dnATM2 & hP53 >= dnP53 & hMDM2 >=
dnMDM2 wait {dhATM=-1,dhP53=-1,dhMDM2=0}
when hATM = dnATM2 do { hATM'= 0, k'=k+1} goto loc_111;
--when hP53 = dnP53 do {hP53'= 0, k'=k+1}  goto loc_201;
end
var
init_reg, acces,
portrait,fstate,nes_cyc_length,pln_cyc_length,fixpoint,r_ini,r
_old,r_new,r_acc: region;

--var
  -- init_reg, acces : region;
init_reg := loc[auto] = loc_101 & hATM>=0 & hP53>=0  &
hMDM2>=0  & hP53 <= dpP53 &  hMDM2 <= dpMDM2;

-- -------------------Les variables--------------------------
acces:= hide k, n in post(post(post(post(init_reg & k=n) &
~k=n))) endhide;
init_reg:=hull(acces) & init_reg;

print hide hATM,hP53,hMDM2  in init_reg endhide;
acces:= hide k, n in post(post(post(post(init_reg & k=n) &
~k=n))) endhide;
init_reg:=hull(acces) & init_reg;
print hide hATM,hP53,hMDM2  in init_reg endhide;

acces:= hide k, n in post(post(post(post(init_reg & k=n) &
~k=n))) endhide;
init_reg:=hull(acces) & init_reg;
print hide hATM,hP53,hMDM2  in init_reg endhide;

-- Analysis commands
--r_ini:=loc[auto] = loc_101 & hATM>=0 & hP53>=0  & hMDM2>=0
& hP53 <= dpP53101 &  hMDM2 <= dpMDM2101 hMDM2 >= dnMDM2101;
r_ini:=loc[auto] = loc_101 & hATM >= dnATM  & hATM>=0 &
hP53>=0  & hMDM2>=0  & hP53 <= dpP53 &  hMDM2 <= dpMDM2;

--r_ini:= loc[auto] = loc_100 & hMDM2>=0 & hMDM2 <= dpMDM2100;
-- hP53>=0  & hMDM2>=0 &  hP53 <= dpP53100 &  hMDM2 <=
dpMDM2100;
r_new:=hide k,n in hull (post(r_ini & k=n) & ~k=n) endhide;
r_old:=r_ini & ~r_ini;

while not empty(r_new) and empty(r_new & r_ini) do
```

```
r_old:=r_new;
r_new:=hide k,n in hull(post(r_new & k=n) & ~k=n) endhide;
endwhile;
-- To verify that the initial zone is accessible from itself
if not empty (r_new & r_ini) then
-- if accessible
r_acc:=hide k,n in hull(post(r_new & k=n) &~k=n) endhide;
r_old:=r_ini & ~r_ini; --empty region initialization
while not empty(r_acc) and not r_new=r_old do
r_old:=r_new;
while not empty(r_acc) and empty(r_acc & r_ini) do
r_acc:= hide k,n in hull(post(r_acc & k=n) &~k=n) endhide;
endwhile;
r_acc:=hull(r_acc & r_ini);
r_new:=hull(r_acc & r_new);
r_acc:=hide k,n in hull(post(r_new & k=n) & ~k=n) endhide;
endwhile;
if not empty(r_new) then
prints
"============================================================"
;
prints "Constrained region of the Invariance Kernel in the
zone:";
--print hide h in r_new endhide;
prints
"============================================================"
;
prints
"============================================================"
;
prints "Delay constraintes:";
print  hide hATM,hP53,hMDM2 in r_new endhide;
prints
"============================================================"
;
else
prints "Invariance kernel does not exist from the initial
region";
endif;
else
-- if not accessible
prints "The initial region is not accessible from itself
hence";
prints "there is no initial condition that leads to an
invariance kernel.";
endif;
```