Hybrid Modeling of the p53 and Akt associated Gene Regulatory Network

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Approval

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Abstract

p53 is known as a genome guardian gene as it bears a putative function in the DNA damage associated gene regulatory networks (GRNs). These GRNs integrate many upstream signals to up-regulate p53 and in turn p53 either up-regulates or down- regulates many downstream signals. A formal model was proposed and analyzed using several approaches like model checking, kinetic logic and hybrid modeling. Initially, the GRN comprising of the entities p53, Akt and Mdm2c and Mdm2n was modeled using kinetic logic of René Thomas. The logical parameters for the qualitative model were infered using the model checking approach implemented in SMBioNet. The qualitative model predicts a stable state and cycles representing the over expression and homeostasis of the entities, respectively. The model also predicts the bifurcation states, which cause divergence from the normal cyclic behaviours towards the stable state. We also computed the conditions in the form of delay constraints for the existence of cycles using hybrid model checking. This model increases the understanding of p53 regulatory mechanism through which the system diverts from normal state to disease state. Thus, the presented model clearly depicts the significance of Akt mediated regulation of p53 by revealing the dynamics involved in the network.

Certificate of Originality

I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any degree or diploma at National University of Sciences & Technology (NUST) Research Center for Modeling and Simulation (RCMS) or at any other educational institute, except where due acknowledgement has been made in the thesis. Any contribution made to the research by others, with whom I have worked at NUST RCMS or elsewhere, is explicitly acknowledged in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except for the assistance from others in the project's design and conception or in style, presentation and linguistics which has been acknowledged.

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Chapter 1

Introduction

1.1 Background

Cancer is the second leading cause of the mortality worldwide and its incidence is predicted to reach 22 million [58]. Cancer is a complex disease and its progression involves a series of genetic irregularities (mutations) which progressively transform normal cells into tumor cells. These tumor cells can invade surrounding tissues and become malignant with accumulating genetic anomalies. Though, early prognosis of cancer holds greater possibility of treatment, detecting it in early stages is still a challenge for the biologists [13]. This challenge can be certainly attributed to the complexity in the underlying signalling pathways involved in the evolution of tumor.

The genomic instability associated with certain types of cancer is detected through tumor markers, which indicate aberrantly expressed genes. Each type of cancer can be identified by unique tumor markers (gene expression). However, the altered expression profile of some genes is common to all types of cancer. Akt and TP53 (gene symbol of p53 protein) are two such genes which are altered in several types of the initial tumors [11]. Diverse research groups have reported overexpression of Akt and mutated p53 in progression of tumor development [44] [13]. Such types of genes are especially important as they are not only used as tumor markers for detection of cancer at early stages but may also become therapeutic targets. This

study aims to analyse and elucidate the underlying dynamics of p53 and Akt associated Gene Regulatory Network (GRN). Significance of this study lies in the fact that the GRN deployed in this study is a novel regulatory network (discussed in section 1.2 in detail) and its dynamics are not established yet. This study conducted an analysis of the p53-Akt associated GRN using various formal modelling approaches (discussed in Chapter 3) to predict its dynamics involved in the evolution of tumor. The details of the p53-Akt associated GRN are discussed below.

1.2 Identification of p53-Akt associated Gene Regulatory Network

p53-Akt associated GRN deployed in this study is a literature curated extended model of ubiquitinated p53 regulation. p53 regulation in physiological and stress conditions is controlled through several mechanisms whose relative contributions may vary among different cell types either normal and/or malignant cells, depending on the stress intensity [38]. We in this study combined two such regulatory mechanisms reported separately in the literature [1], [8]. An integrated overview of this model is presented in the following sections below.

1.2.1 p53 associated Gene Reguatory Network

p53 is a transcriptional regulator of a large number of genes involved remarkably in growth arrest, DNA repair, apoptosis and cellular senescence [23]. The ability to control cell proliferation makes p53 an exciting therapeutic intervention for the treatment of cancer [21]. The mutations in p53 have been reported to act through other protein substrates which in turn accelerate the pleiotropic effects on cancer cells [32].

p53 is a tumor suppressor protein. It is encoded by TP53 gene located on the short arm of chromosome 17 (17p13.1) [16] in humans. Structural organization of p53 consists of several conserved domains notably an acidic N-terminus transcription-activation domain (TAD), activation domain 2 (AD2),

proline rich domain, central DNA-binding core domain (DBD), nuclear localization signaling domain, homo-oligomerisation domain (OD) and C-terminal domain [22]. Each domain plays a critical role in the regulation of many important genes which are JNK1-3, ERK1-2, p38 MAPK, ATR, ATM, CHK1, 2 and TP53. Such diversity of downstream effector proteins of p53 depicts its significance in cell cycle control.

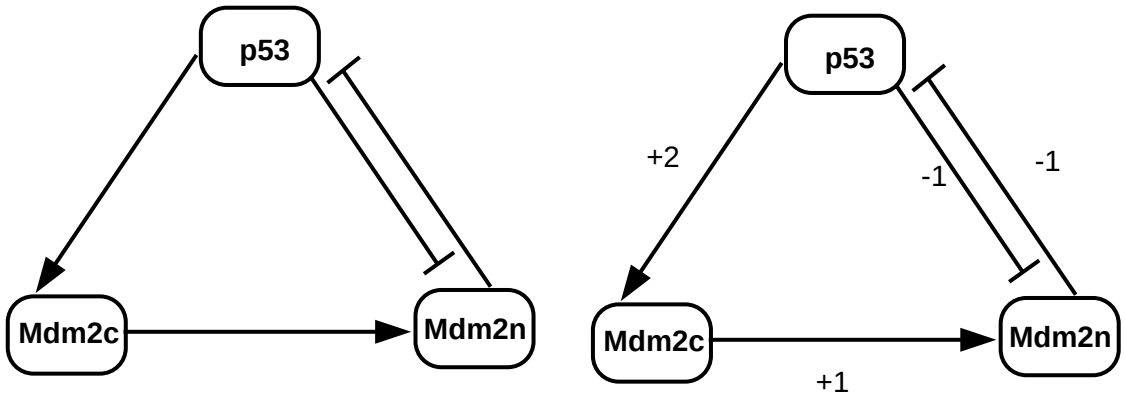
In normal cellular conditions, p53 level remains low and its concentration increases only when cells undergo certain stress conditions, for example by ionizing radiations, DNA damage, abnormal growth signals or drugs [1]. Mouse double minute 2 homolog (Mdm2) belongs to an E3 ubiquitin-ligase protein family and is itself a p53 transcriptional target gene. Mdm2 is present in the cells in two forms i.e., cytoplasmic and nuclear. Mdm2 tightly regulates p53 level through a negative (both cytoplasmic and nuclear Mdm2) and a positive (involving nuclear mdm2) feedback loop to maintain the homeostatic conditions of the cell (Figure 1.1(a)). Degradation of p53 by Mdm2 occurs through either ubiquitination [13] or by blockage of p53 transcriptional activity [18]. The oscillatory behaviour of p53 in normal cells is thus attributed to all such regulatory mechanisms [1].

In stressed cellular environment, several pathways cause disruption of Mdm2 and p53 complex which subsequently increase the level of active p53 in the cell. The resultant high concentration of p53 triggers cellular repair process or the synthesis of pro-apoptotic proteins, thereby preventing the proliferation of genetically unstable cells [1].

Accumulating evidence have identified impaired p53 regulatory network in early stages of cancer [54]. Previously, established kinetic models of p53 regulation [1] [13] have accounted only the ubiquitination process to demonstrate its dynamics. Figure 1.1(a) shows one such gene regulatory network [1]. This network is taken from [1] and is composed of three elements i.e p53, cytoplasmic and nuclear Mdm2. The logical caricature of this network as established by [1] is shown in Figure 1.1(b). Two feedback loops are present in this network.

1. A positive feedback between p53 and Mdm2n.

2. A negative feedback loop involving p53, Mdm2c, Mdm2n and p53.



(a) (b)

Figure 1.1: **Ubiquitination based regulation of p53 adopted from** [1]. The p53 gene regulatory network represents a model presented [1]. (a) Interaction graph of p53 is presented: where normal arrows corresponds to positive interaction and blunt arrows to negative interaction. (b) Logical regulatory network of p53 GRN as described [1]. The labels of the edges indicate the sign of interactions and the order of their threshold.

The intracellular protein levels of p53 and Mdm2 have been studied to oscillate variably under different stress conditions using this model [10]. The qualitative analysis of p53 regulation model (adapted from and shown in Figure 1.1) presented in [1] demonstrated the oscillatory behaviour of p53-Mdm2 complex under various conditions for example dose dependent irradiation response, failure to respond to irradiation, shifts in the frequency of oscillations and dampening of the oscillations. Therefore, this network represents a good starting point for studying extended dynamics of p53 regulation.

1.2.2 p53-Akt associated Gene Regulatory Network

Mutations in both p53 and Akt represent the most common genomic changes in many types of cancer especially at early stages [44]. The molecular dynamics involved in their mutual deleterious effects on tumor evolution

are yet to establish. Therefore, we in this study took a step forward and extended the p53 ubiquatination regulatory model of p53 (Figure 1.1) by including Akt in it and predicted the mutual effects of p53 and Akt on the tumor evolution.

Akt also known as protein kinase B (PKB) belongs to a serine/threonine protein kinase family and plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. The Akt signaling pathway leads to cell survival (blocking apoptosis) [45]. Various growth factors, hormones, and cytokines activate Akt by binding to their cognate Receptor Tyrosine Kinase (RTK), cytokine receptor, or G protein-coupled receptors (GPCRs), which mostly occurs downstream of phosphoinositide 3-kinase (PI3K) [8]. PI3K mediate its signaling through a diverse array of receptors by phosphorylating membrane phospholipid PtdIns(3,4,5)P_2 (PIP2) to generate PtdIns(3,4,5)P3 (PIP3), which cause translocation of PH domain of Akt to the plasma membrane. Subsequent translocation leads to the complete Akt activation by phosphorylation through phosphoinositide-dependent kinase-1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2) in two amino acids residues i.e, threonine 309 and serine 474, respectively [9] . Activated Akt protein kinase then targets specific substrate proteins in the cytoplasm and nucleus [10].

With the discovery of the persuasive contribution of PI3K and Akt activation to tumorigenesis, intense research into the regulation of this pathway has emerged. One such contribution involves the demonstration of Akt signalling pathway in p53 regulation [24]. While, the oscillatory behaviour of p53-Mdm2 regulation in response to ionizing radiation and/or stress is well established, the physiological significance of such oscillations remained unclear until [1] demonstrated the physiological occurrence and combined effects of PI3K amplification and loss of p53 mediated regulation through Akt pathway (shown in Figure 1.2 and adapted from [8]). Their work revealed that the negative feedback loop of p53-Mdm2 complex is embedded in a network involving a mutual antagonism (or positive feedback loop) between p53 and Akt which can be considered a putative cause of this oscillation under physiological conditions.

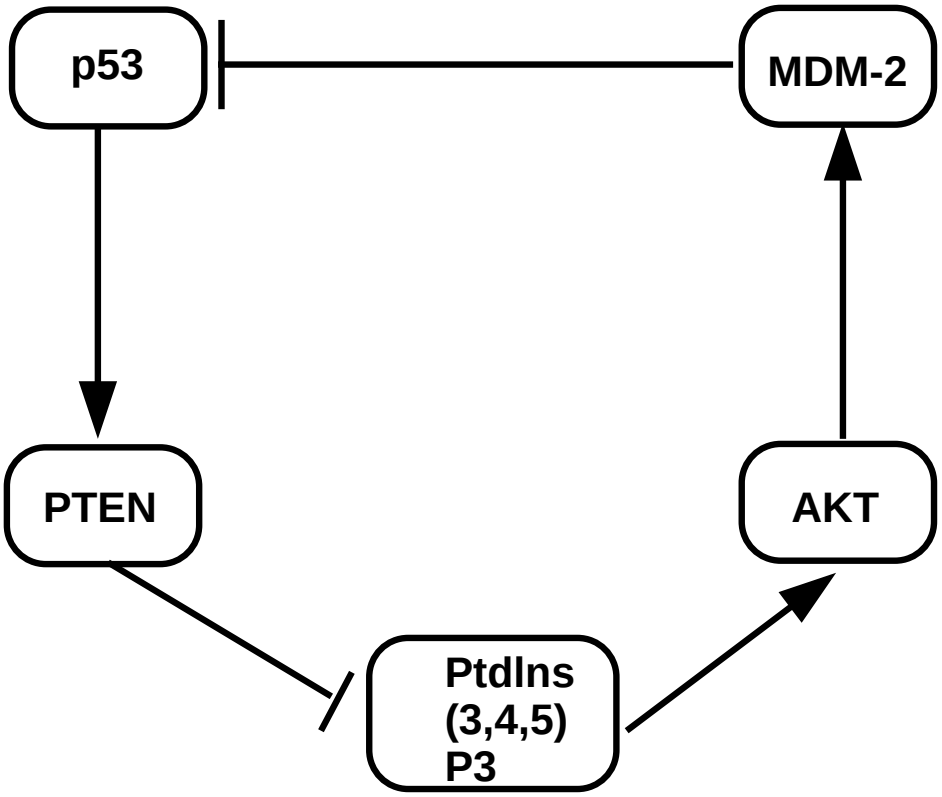


Figure 1.2: **The p53-Akt associated GRN adopted from** [8]. p53 positively regulate PTEN levels, which reverse P13K action by dephosphorylating Ptdlns(3,4,5)P3. Ptdlns(3,4,5)P3 recruits Akt to the membrane, where Akt becomes phosphorylated and activated. In turn, activated Akt phosphorylates Mdm2 which leads to the nuclear import of Mdm2. In the nucleus, Mdm2 binds to and degrades p53. Each entity is presented inside a rounded box. Normal arrows corresponds to the activation and blunt arrows corresponds to the inhibition.

Figure 1.2 shows the physiological regulatory mechanism of p53 regulation through Akt as demonstrated in [8]. This molecular mechanism shows the involvement of Akt in the regulation of p53 represented with Akt$\rightarrow$ Mdm2$\rightarrow$ p53 interactions.

The extended model used in our study which is constructed from combining the gene regulatory models presented in Figures 1.1 and 1.2 is illustrated in Figure 1.3. This p53-Akt associated GRN shows an all-or-none switching

behaviour between a pro-survival cellular state (low p53 and high Akt levels) and a pro-apoptotic cellulare state (high p53 and low Akt levels) [57]. This GRN possess three feedback loops as compared to the other two GRNs (as shown in Figure 1.1 and 1.2 respectively). Essential proteins interactions which are involved in the p53 associated GRN are summarized as follows:

1. Nuclear Mdm2 down regulates p53 level and is itself up regulated by cytoplasmic Mdm2 (nuclear entry).

2. p53 exerts a positive control on the level of cytoplasmic Mdm2 (transcriptional activation) and a negative control on the level of nuclear Mdm2 (blockage of Mdm2 nuclear entry).

3. Mitogen-induced activation of PI3K and its downstream target Akt, results in phosphorylation of Mdm2c on serine 166 and serine 186. Phosphorylation on these sites is necessary for translocation of Mdm2 from the cytoplasm into the nucleus. Therefore, the expression of constitutively active Akt promotes nuclear entry of Mdm2, diminishes cellular levels of p53, and decreases p53 transcriptional activity.

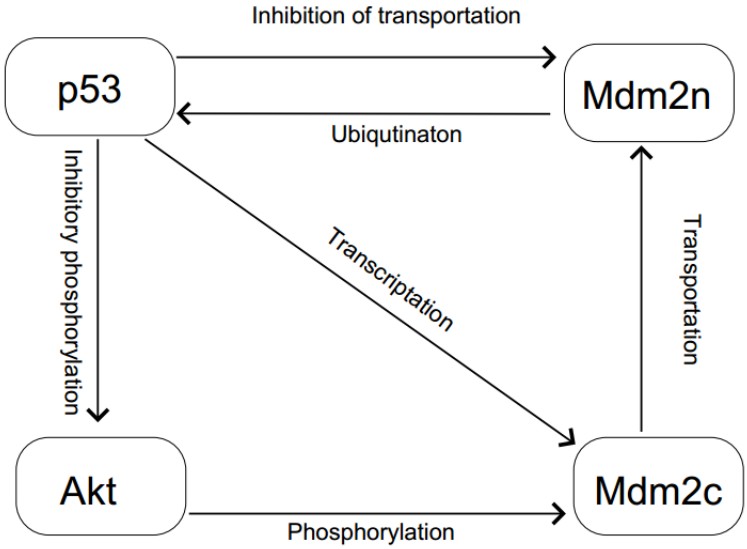


Figure 1.3: **The extended p53-Akt associated Gene Regulatory Network (GRN).** Figure shows the entities, transitions biological process involved in the regulation of p53 (a tumor supressor gene). p53 is regulated via three feedback loops here: A negative feedback loop which involves Mdm2n, p53 and Mdm2c, a positive feedback loop which involves Akt, Mdm2c and Mdm2n, and a feedback loop between Mdm2n and p53.

p53 and Akt influence the process of cell apoptosis and survival in entirely opposite ways. As mentioned earlier p53 regulate genes involved in the cell cycle arrest and apoptosis. In response to DNA damage, p53 is imported into the nucleus, and then binds it to the target genes and alters their transcription to start cellular repair process and shifts the cell into pro-apoptotic state [8]. Low levels of Akt gene expression are observed in the presence of high levels of p53 [24].

On the other hand, high level of Akt in cells activate survival signals and stimulates biological pathways which then restricts level of p53. Thus, Akt plays a crucial role against apoptosis and promoting survival [39]. Akt has been reported to cause malfunctions in cellular repair or apoptosis that leads to cancer or cellular mutations at its maximum threshold [17].

In short extended model of Akt-p53 associated GRN in this study could demonstrate useful insights into how this pathway, which is inappropriately activated in many malignancies [37], affects the function of p53 and might contribute in understanding the tumor physology under such condition.

1.2.3 Cancer Research scenario in Pakistan

Detecting cancer in early stages is still a challenge for biologists and poses serious health threats at national level. Our study aims to elucidate the dynamics of a p53-Akt associated GRN involved by presenting a formal model followed by critical analysis. Therefore, underlying research will be helpful in several areas including reliable target identification for cancer drugs and early cancer diagnosis. It will also assist in further academic cancer research.

1.3 Thesis Contribution

The present study presents the qualitative and hybrid modeling of the p53 and Akt associated gene regulatory network (GRN) which is the extension of the GRN presented in [1]. Several novel predictions regarding the role of p53 and Akt in tumor evolution are revealed using several formal modeling and analysis formalisms which include multivalued Kinetic Logic of Rene Thomas [6], Computational Tree Logic [33] and Bio-LHA[7]. We started with the construction of the qualitative model of p53-Akt associated GRN which is composed of four entities i.e., p53, Mdm2n, Akt, Mdm2c. The logical parameters for the qualitative model were inferred using the model-checking approach implemented in SMBioNet. The qualitative model showed the stable state and qualitative cycles. The stable state predicted the over-expression of the entities Akt, Mdm2n and Mdm2c and the suppression of p53 while the cycles predicted the homeostasis of the all the entities of the GRN. The bifurcation states were found more in those cycles where activated Akt appears in more number of states than P53. The qualitative model was further enriched with time delays as unvalued parameters and then this refined hybrid model was analyzed for the unvalued delay constraints representing the existence of the

qualitative cycles. These delay constraints suggest that the cycles representing homeostasis (resp.do not exist) for those values of delays which satisfy (resp.violate) them.

1.4 Organization of the Thesis

This thesis is comprehensively written in five chapters. Chapter 1 provides a comprehensive background of an introduction to the research area, an overview of the problem, and the research contributions. Chapter 2 comprehensively summarizes the literature review conducted to identify the challenges and define the problem statement. Chapter 3 discusses the methodology used for modeling of the selected gene regulatory network (GRN). Results are presented in chapter 4 along with a discussion. Chapter 5 concludes the research presented in the dissertation and discusses some future recommendations.

Chapter 2

Literature Review

The present research adapted a well recognized technique of hybrid modeling for Gene Regulatory Networks (GRNs) [6]. Primarily using the discrete approach of René Thomas, a qualitative model of p53-Akt associated GRN was constructed. Secondly the hybrid model was derived by introducing time delays [6].

Experimental approaches to study living system behaviors normally focus on various complementary biological components. Schlitt and Brazma, [43] found that it is not enough to study the individual genes, proteins and RNA at a time. So, they suggested to study behaviours of entities in a cell in the form of interactions [43]. A group of such interactions is called Biological Regulatory Network (BRN). Among these BRNs, GRNs are specifically important because the rates at which genes are transcribed into mRNA and the way in which genes interact with each other (through RNA and protein products) and with other substances in the cell is controlled by a GRN. A GRN provides the major biological framework for examining dynamical biological behaviors [35].

2.1 p53-Akt associated GRN: Implications in Cancer

GRN associated with the DNA damage response holds p53 tumor suppressor gene as a main regulator of cellular signalling in response to stress [36]. This protein is a transcriptional regulator of a numbers of genes remarkably associated with apoptosis, DNA repair, growth arrest and cellular senescence, making it a vital component to hinder the proliferation of irregular cells. It brings different cellular outcomes including apoptosis and cell cycle arrest [9] to remove damaged cells and aid in DNA repair process. It has been well-established that p53 tumour suppressor protein has a key role in halting the spread of cancer in several human malignancies [16]. The ability of this important tumour suppressor protein is strongly synchronized with the ubiquitin ligase Mdm2 through a negative feedback route. This negative circuit destabilizes the high occurrence levels of p53 that would be deleterious for cells [16] under normal physiological conditions, thus keeping the level of p53 low. p53 raises its level only when cells are damaged or stressed, e.g., by ionizing radiations, drugs or aberrant growth signals. Specifically, the DNA damage caused by ionizing radiations increases the level of p53 activation which in turn activates the synthesis of pro-apoptotic proteins and initiate the damage repair process to prevent the proliferation of unstable cells genetically [1]. According to a number of studies up to 50% of human tumours result due to changes in the p53 gene position or its altered expression [11].

Mitogen-induced activation of phosphatidylinositol 3-kinase (PI3-kinase) and its down-stream target, the Akt/PKB serine-threonine kinase, phosphorylats Mdm2 at serine 166 and serine 186. Phosphorylation on these sites is essential for translocation of Mdm2 from the cytoplasm into the nucleus [14]. Appearance of constitutively active Akt promotes nuclear entry of Mdm2 and reduces cellular levels of p53 and decreases p53 transcriptional activity. It shows that PI3-kinase/Akt signalling modify Mdm2 localization provides a close overview of how this pathway is inappropriately activated in most malignancies could modify p53 functions [37].

Another frequently mutated tumor suppressor gene found in a variety of

human cancers is PTEN and it is a negative regulator of PI3K/PKB Akt-dependent cellular existence [15]. Investigation of the human genomic PTEN locus showed a p53 binding element, directly upstream of the PTEN gene. Mutation and deletion analyses showed that this part is essential for inducible transactivation of PTEN through p53 (besides other p53-independent factors controlling its expression). The induction of p53 in wild-type and tumour cell lines showed enhanced levels of PTEN mRNA [24]. p53-mediated apoptosis occurs in the presence of PTEN, demonstrated in immortalized mouse embryonic fibro-blasts. These findings drags our attention towards the role of p53 in the regulation of cellular survival by the virtue of its tumour suppressor abilities [46].

There exists conflicting signals between Akt and p53 integrated in a network, transducted through two different pathways [24]. Cross talk between Akt and p53 forms a positive feedback loop that comprises within itself a negative feedback loop between p53 and Mdm2 [28]. These united circuits are associated with the tumor suppressor-oncoprotein networks in such a way that a positive response loop exists within p53-Mdm2-Akt-p53 and a negative response loop exists between p53-Mdm2-p53. Wee and Surana in 2006, stated that the switch between these positive and negative feedback loops determines the cell fate, i.e., entry into a pro-survival or apoptotic state [56]. The same research group in a later study in 2009, found that these oscillations are connected with a major decrease in the threshold level of IR at which switching from a pro-survival to a pro-apoptotic state takes place. The expression level of p53-target genes is affected by the oscillatory or non-oscillatory behaviour of p53 to subsequently carry out cell cycle arrest/DNA damage repair against apoptosis or growth [57].

Akt may not be necessarily down regulated by apoptotic process on one side, while it tends to activate Mdm2 (key regulator of p53) upon serum stimulation on the other side. Gottieb and Leal in 2002, proposed that the phenomenonas of apoptotic cell death carried out by p53-dependent down regulation of Akt and generation of survival signals (achieved through Akt) to direct the activation of Mdm2, inhibition of p53 and subseqent inhibition of p53-dependent apoptosis are governed by the balance of signals [24].

p53 pathway responds to environmental and genetic stress signals by interrupting the processes of DNA replication and cell division [19]. Upon receiving a stress signal, p53 initiates its transcriptional activity and several programs like cell cycle arrest, apoptosis and cellular senescence start. p53-responsive genes generate proteins that interact mutually with a huge number of other signal transduction pathways in the cell and a number of negative and positive response loops can alter p53 mediated activity [55]. Six feedback loops operate through MDM-2 protein to control p53 activity. p53 shares information with the Wnt-beta-catenin, p14/19 ARF, p38 MAP kinase, IGF-1-AKT, cyclin-cdk, Rb-E2F, p73 gene and the cyclin G-PP2A gene products. The activity of p53 is regulated by at least three different types of ubiquitin ligases that act in an auto regulatory way: MDM-2, Pirh-2 and Cop-1 [27]. Thus, it is important to deeply examine the signal transductions involved in p53 pathway to develop a better understanding of cancer mechanism.

The activation of p53 accompanied by oncogenic Ras leads to cell cycle arrest or cellular senescence [42]. Ruiz in 2008, analysed the phenomenas of cell cycle arrest or senescence with the help of two different qualitative and qualitative models, i.e., whether the level of p53 or non-qualitative factors (e.g., tissue origin or cells genotype) influence its biological outcomes [34]. For this purpose, different situations were compared that brought senescence and found that Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PPP1CA) stimulated by Ras is the factor that contributes to Ras-induced senescence (mainly through pRb dephosphorylation inhibition). This shows that cycle arrest or cellular senescence do not depend upon levels of p53 activation whereas it contributes as a necessary condition for this process to occur [42].

Several genetic mutations result in inactivation of p53 leads towards overexpression of PI3K and lethally uncontrollable growth of cells [8]. It is illustrated in several studies that Akt inactivation involves several PTEN-independent and dependent pathways, whose relative contributions may vary among different cell types as well as between malignant and normal cells and are based upon the stress concentrations [38].

2.2 p53-Akt based mathematical models and Observations

To understand thoroughly, how the structure of a network defines the functional behaviour of these systems is still challenging and requires more research. For this purpose, numerous modelling approaches were used to study the dynamical behavior of p53-Akt network both quantitatively [25], [40] and qualitatively [57], [4], [48].

Mathematical models suggest that under certain circumstances oscillations in Mdm2 and p53 protein levels occur in response to a stress signal. A delay in p53 dependent stimulation of Mdm2 is expected to be essential although not sufficient for this oscillatory behaviour [47]. Upon contact of different cell types to ionizing radiations, the cellular levels of Mdm2 and p53 oscillates. These oscillations tend to repair DNA without unnecessary activation (production) of p53 in the cells [10] and maintains the level of p53 concentration in a highly regulated fashion [59]. Bar-Or and co-workers presented a methematical model of p53-Mdm2 interactions and found that Mdm2 induced by p53 requires a delay to exhibit an oscillatory behavior and the period of this oscillation is determined by the length of the delay. On the basis of this finding it was concluded that it is important to keep an intermediate range of p53-Mdm2 interactions to generate oscillations [10].

A discrete model of p53-Mdm2 was constructed by Ahmad et al. (2011) to study the role of p53 activation upon stress (ATM) induction. They applied qualitative logical formalism that generates all the possible states of a BRN. It helps the researcher to gain important biological inference by observing homoeostatic cycles and bifurcation points. Their model revealed a stable steady state with high Mdm2 expression while oscillatory behaviour of p53 was exhibited that represented homoeostatic level of genes after the arrival of stress conditions [4].

Abou-Jaoudé et al. (2009) made a differential model based on the antagonistic circuit between p53 and Mdm2 that encompasses a negative circuit between p53, Mdm2 (they reformulated the interactions by introducing cytoplasmic and nuclear Mdm2). On one hand, cytoplasmic Mdm2 is positively

regulated by p53 while on the other hand, nuclear Mdm2 is negatively regulated by p53. Small number of bifurcation analysis were presented that focused on the oscillations between p53 and Mdm2. Their model showed a great variability of behaviour depending upon the transactivation of p53 and post-translational modifications that vary between different cell types [1].

Hui in 2007, developed a model to study the dynamical mechanism of the negative feedback loop comprising of Mdm2 and p53 proteins regulated by p14/19ARF and numerically tested the system with different parameter values to observe its respective behaviour [55]. Specific assumptions characterizing the interactions between Mdm2 and p53 regulated by p14/19ARF directed an oscillatory performance of Mdm2, p53 and p14/19ARF protein levels after a sufficiently strong damage signal. These oscillations exhibit execution of a reversible p53 response in a more effective manner [31].

A dynamical model of p53-Mdm2 feedback regulated by Akt was given by Chang-qing and co-workers in 2009. The model showed stable oscillations between p53 and Mdm2 that is essential to carry out the process of apoptosis or cell repair. This model also revealed oscillation-stationary bifurcation for p53 kinetics that indicated the switch between death and survival [19]. The model simulation indicated that level of Akt activation would decide whether to keep p53 at a low expression level or to bring cellular repair or apoptosis, i.e., low Akt activation level would not hinder p53 dependent apoptosis or cellular repair but high Akt activation level kept p53 at a low expression level. This would result into malfunctioned cellular apoptosis or repair mechanism making the cell susceptible to possible mutation or cancer in future [17].

The logical parameter collection step for modeling process holds a main position in describing the properties of a GRN. An extension to René Thomas discrete modeling framework was extended by Khalis et al. (2009) that encoded multiplexes information on cooperative, concurrent or more complex molecular interactions, and highlighted that how formal methods help in the parameter selection step. For this purpose, they used temporal logical formula to express dynamical knowledge and validated by using model checking algorithm. This new formalism was implemented in SMBioNet software. [33].

BRN modelled in SMBioNet tool uses Computation Tree Logic (CTL)

and the multivalued logical formalism of René Thomas. Information is given in the form of a discrete regulatory network and a CTL formula (that expresses the biological observations). An Output is generated comprising all the parametrizations of the network that guide to a dynamical model satisfying the given CTL formula. The verification step is achieved with the NuSMV symbolic model checker [33].

In 2012, Ahmad and Niazi applied modelling formalism of René Thomas for modelling and analysis of MAL-associated BRN in case of cereberal malaria (CM). The set of parameters was carefully selected which reflect the dynamics of the BRN. These parameters are primarily unknown but can be inferred from the properly chosen observed dynamics of a BRN. The behavioural analysis of discrete model was further enhanced by using the model checking software HyTech that employed delay parameters. They found different trajectories and essential conditions that can cause the pathogenesis of CM and predicted that hyperinflammation would occur if Brutons tyrosine kinase (BTK) remains constitutively active accompanied with high dosage of cytokines present in the cellular environment. The formal modelling and analysis paves the way to correctly analyse a BRN and to make calculations about essential trajectories which shows the way to a diseased or normal response [6]. The aim of computational modeling is to relate its results to wet-lab experiments by supplying model arrangement methods to biologists and model justification tools from active research in theoretical computer science. These resulting formal models are not simply the explanations of biological results but also act as guide lines for biological experiments [12].

2.3 Computational Modeling of p53-Akt associated GRN

GRNs have a vital role in every development of life, as well as metabolism, cell differentiation, signal transduction and the cell cycle. By accepting the dynamics of these networks we can drop light on the methods of diseases that occur when these cellular progressions are deregulated. In the current

study, we implemented a hybrid model of p53 associated gene through computational techniques. Previously this has only been done through wet-lab experiments. Accurate prediction of the behaviour of p53 regulatory network in cancer diagnosis will speed up biotechnological understanding and identification of the current stage of disease and also help in taking early measures to stop the spreading and enlargement of cancerous tissue; as such guesses are cheaper and quicker than lab experiments. Computational methods, both for supporting the development of network analysis and for the models of their functionality, have already proved to be a precious research tool.

To understand the dynamics of p53-Akt network and for its optimization, a computational approach was used that applied a CTL behavioural assessment. This approach predicts targeted information about the diseased and recovery states of cancer that were actually not identified previously. This systematic way will save the excessive experimental time as well as its cost.

Chapter 3

METHODOLOGY

This chapter introduces the methodology used in this thesis for the modeling of Gene Regulatory Networks (GRNs). Three different modeling frameworks have been used for the modeling and analysis of GRNs which include Kinetic Logic of René Thomas [6], Computational Tree Logic (CTL) [33] and Bio-LHA [2]. The integrated work-flow based on these formalisms is presented in Figure 3.1. The work-flow starts with the construction of a qualitative model of GRN using Kinetic Logic of René Thomas described in section 3.1.1. The qualitative model is then verified using model checking formlism i.e. CTL described in section 3.2. For this purpose, biological observations regarding the behaviour of biological entities are extracted from literature and used as a verification critreia. The literature curated biological properties are translated into CTL formulas and formally checked against the possible model state space. In SMBioNet tool the correct models which satisfy these biological properties are selected for further analysis.The selected model is further converted into a hybrid model using Bio-LHA (explained in the section 3.3) and dynamics of GRN are computed.

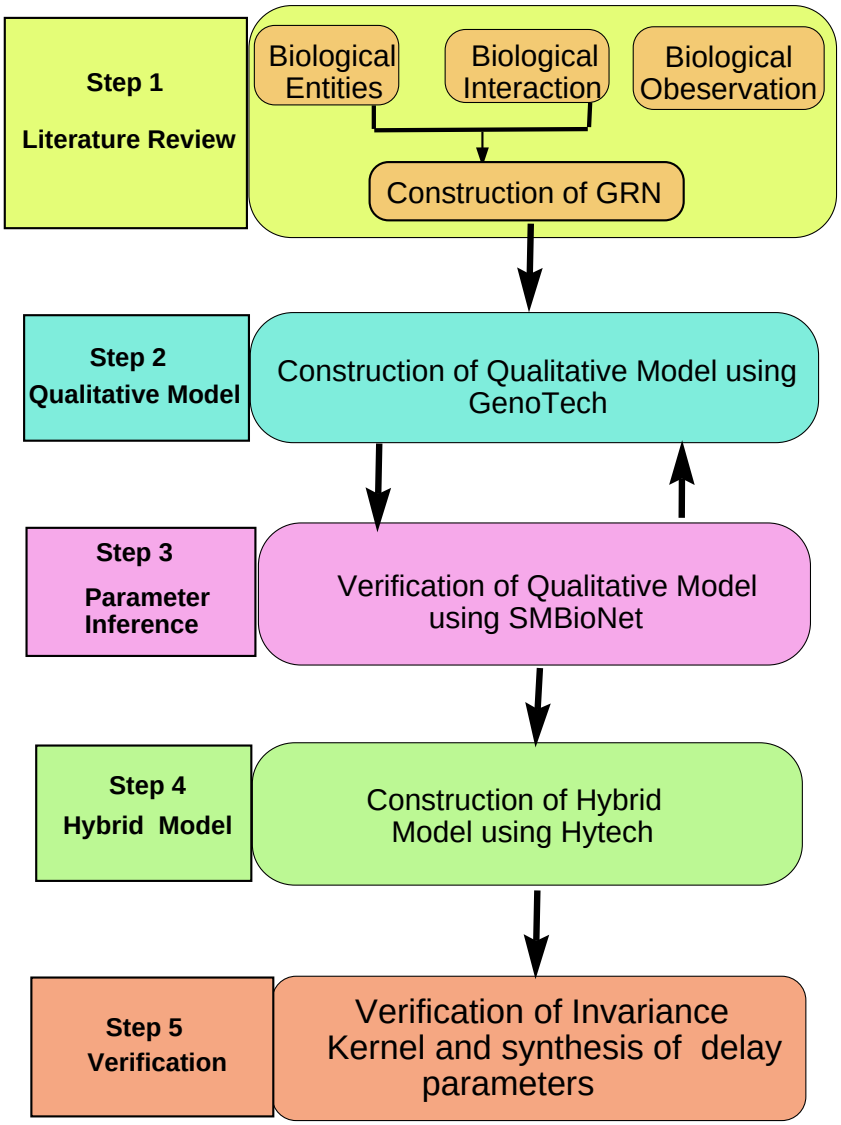


Figure 3.1: **Methodology for the study.** This study starts with literature review for construction of the GRN. It is proceeded by qualitative modeling, parameter inference and hybrid modeling. It ends with the verification of invariance kernel and synthesis of delay parameters.

3.1 Qualitative Modelling

The qualitative modeling defines a system in terms of qualitative properties and offers an abstraction of the quantitative reasoning. It is especially well-suited to model the biological systems due to their inherent complexity and scarcity of the relevant quantitative data. The "gene expression" is a complex process with dynamic behaviors. The dynamics of gene expression cannot be predicted without the help of computational methods with the ability to represent it in the form of a regulatory network. The qualitative modeling framework adopted in this work i.e. the Kinetic Logic of René Thomas has the ability to represent the biological regulation as a graph and carrying out the dynamic analysis based on Kinetic Logic [50],[53],[51]. Initially, this framework was based on Boolean Logic which takes two values for each entity which can be either present (1) or absent (0). Later, René Thomas improved this framework by deployment of Generalized Logic instead of Boolean Logic to cater the realistic gene expression, which is multivalued [53]. This framework is also called multivalued logics.

3.1.1 Approach of René Thomas

The formal semantics of Thomas' formalism for the modeling of GRNs is explained in this section. In this framework, biological regulation is based on kinetic logic which focuses threshold parameters of its entities (genes/proteins). Biological regulation between two entities of a GRN can be seen as either activation or inhibition. Figure 3.2. represents sigmoid curves and logical (discrete) to illustrate the activation and inhibition of a hypothetical gene expression, respectively. Expression of a gene *"b"* under positive (respectively negative) influence of gene *"a"* will tend to increase (respectively decrease) the synthesis of gene b, as shown in Figure 3.2. A and C (respectively Figure 3.2. B and D).

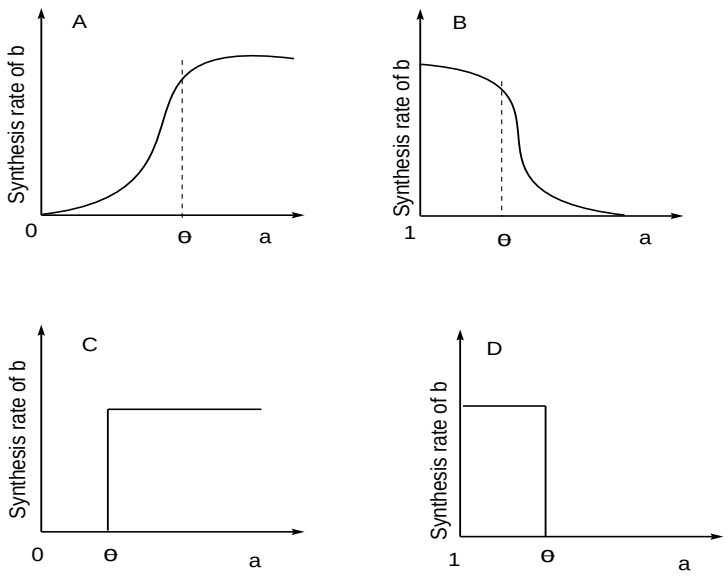


Figure 3.2: **Hypothetical Gene Evolution Curves** Sigmoid graphs A and B represent activation and inhibition as increasing and decreasing sigmoid, respectively. C and D represent the discrete approximation of A and B, respectively. θ represents the thresholds of regulation.

We will use a GRN of the mucus producing pathogen, "Pseudomonas aeruginosa" as an example to illustrate Thomas' formalism. Pseudomonas aeruginosa is a common opportunistic bacteria which secrete mucus in chronic lung diseases such as cystic fibrosis. The regulatory network of the mucus production has been explained in [26]. The simplified mucus production GRN in its simplified form is shown in Figure 3.3.

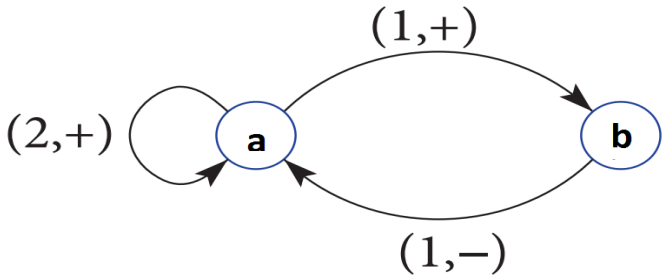


Figure 3.3: **A GRN of Pseudomonas aeruginosa**. A Pseudomonas aeruginosa is an example of a GRN where a and b represent biological entities. The labels +, - and 1, 2 represent the activation, inhibition and the threshold concentration respectively.

The GRN of Pseudomonas aeruginosa consists of AlgU (var. a) and its inhibitor protein (var.b). AlgU activates its inhibitor at lower threshold than its own activation threshold. The formal semantics of Kinetic logic, adapted from [6],[52], are illustrated below using Pseudomonas auerignosa GRN as a running example.

Definition 3.1 (Directed Graph) *An ordered pair G(N,E) is called a directed graph, where*

- *N is the set of all nodes and*

- $E \subseteq N \times N$ *is the set of ordered pairs called edges or arcs . In a directed graph G(N,E),* $G^{+}(x)$ *and* $G^{-}(x)$ *denote the set of successors and predecessors of a node* $x \in N$*,respectively.*

Figure 3.3 shows Pseudomonas aeruginosa GRN represented by a directed graph showing the genes a and b , where N={(a,b)} and E={*(a,b), (b,a), (a,a)*} or the set of genes and edges respectively. In case of gene a, $G^{+}(a) = \{a, b\}$ and $G^{-}(a) = \{b\}$ represents its successor and predecessor respectively.

Definition 3.2 (Gene Regulator Network) *A GRN is represented as a labelled directed graph, where each node corresponds to the biological entities (gene, proteins) and each edge represents the biological interaction between these entities.*

Each edge $a \rightarrow b$ is labeled by a pair (j_{ab}, η_{ab}), where η_{ab} is either the (+) or the (-) which corresponds to activation and inhibition respectively; j_{ab} should be a positive integer corresponding to qualitative level (representing a threshold). For an example, see Figure 3.3, where j_{ab} *=1,* j_{ba}*=1,* j_{aa}*=2* and similarly $\eta_{ab} = \text{“}+\text{”}$, and $\eta_{ba} = \text{“}-\text{”}$, $\eta_{aa} = \text{“}+\text{”}$. To understand the dynamics of a GRN, it is important to take into account all the possible number of states and transitions between them.

Definition 3.3 (States) *The state of a GRN is a tuple* $s \in S$*, where*

$$S = \Pi_{a \in N} Y_a$$

Each qualitative state is represented by vector $(s_{x_a})_{\forall a \in N}$*, where* x_a *denotes the level of concentration of product “a”. discrete levels* Y_a { *0,1,* $\cdots$ *max (*j_{ba}*)*} *where* $b \in G^{+}(a)$

At any instant of time, a qualitative state say *“Z”,* represents the configuration of all elements of a GRN. The dynamics of each entity in a particular state *“Z”,* depends upon its regulators. The number of regulators of a particular variable at a given level of concentration is represented by its set of resources (see the definition of Resources given below). For our running

example, column 1 and 2 of Table: 3.1 represents all possible states (configurations) of genes *"a"* and *"b"* in the GRN of Pseudomonas aeruginosa.

x_a	x_b	R_{x_a}	R_{x_b}	$K_a(R_{x_a})$	$K_b(R_{x_b})$
0	0	{b }	{ }	0	1
1	0	{ b }	{a }	1	0
2	0	{a,b }	{a }	1	2
1	1	{ }	{ a }	0	1
2	1	{ a }	{ a }	2	1

Table 3.1: Table of states (x_a , x_b), resources (R_{x_a} , R_{x_b}) and logical parameters ($K_a(R_{x_a})$, $K_b(R_{x_b})$) of GRN in Figure 3.3.

Definition 3.4 (Resources) *The set of resources R_{x_a} of a variable $a \in N$ at a level x is defined as $R_{x_a} = \{b \in G^-(a) \mid (x_b \geq j_{ba}$ and $\eta_{ba} = "+")$ or $(x_b < j_{ba}$ and $\eta_{ba} = "-")\}$.*

Definition 3.5 (Logical Parameters) *The dynamic behavior of a GRN depends upon the values of logical parameters. The set of these logical parameters is defined as $K(G) = \{K_a(R_{x_a}) \in Y_a \forall a \in N\}$. The parameter $K_a(R_{x_a})$ governs the discrete concentration of evolution of the entity x_a via the evolution operator agsinst the following rules.*

1. *if $x_a = K_a(R_{x_a})$ then x_a cannot evolve from its current level.*
2. *if $x_a > K_a(R_{x_a})$ then x_a decreases by one unit.*
3. *if $x_a < K_a(R_{x_a})$ then x_a increases by one unit.*

Resources represent either the presence of activator or absence of an inhibitor. In case of our running example, the set of resources of genes *"a"* and *"b"* of GRN of Pseudomonas aeruginosa are shown in column 3 and 4 of table 3.1 for each state. It can be seen that there are two resources of gene *"a"*; it is either activated in the absence of gene b or by itself. On the other hand the only resource of gene *"a"* is gene *"b"* which behaves as its activator (being its transcription factor). The logical parameters of the GRN of Pseudomonas aeruginosa (Figure 3.2) are shown in column number 5 and 6 of Table 3.1.

Definition 3.6 (State Graph) *Given s_{a_a} as the concentration level of entity"a" in a state $s \in S$, a state graph of the GRN can be represented as a directed graph G=(S,T), where S denotes set of states and $T \subseteq S \times S$ represents a relation between these states, (called the transition relation) such that $s \rightarrow \acute{s} \in T$ if and only if:*

1. *$\exists$ a unique $a \epsilon N$ such that $s_{x_a} \neq s'_{x_a}$ and $s'_{x_a} = s_{x_a} \Rsh K_a(R_{x_a})$, and*
2. *$\forall\ b \epsilon N \setminus \{a\}\ s'_{x_b} = s_{x_b}$*

The state graph of our running example is shown in Figure 3.4. It has six steady discrete in total along with one stable state (2,1) and a cyclic trajectory {(0,0)$\longrightarrow$(1,0)$\longrightarrow$ (1,1) $\longrightarrow$(0,1)$\longrightarrow$(0,0) }. Thus, from a biological point of view the stable state (deadlock) represent over expression of mucus production as in cystic fibrosis and cyclic trajectory depicts homostasis of the normal function of mucus production in nature.

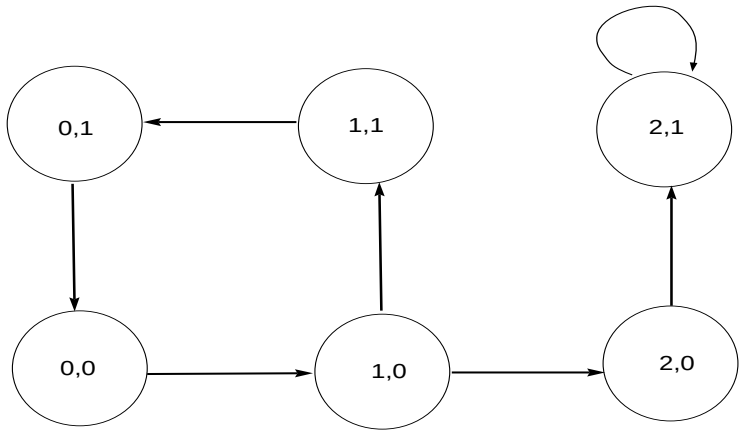


Figure 3.4: **State Graph.** The state graph of the GRN in Figure 3.3. Each node represents a qualitative (discrete) state of the GRN. The values inside a state shows the concentrations of the entities a and b.

There are several discrete modeling tools based on the Kinetic Logic Formalism of René Thomas such as GINsim [2] and GENOTECH [5]. We used GENOTECH [3] for modeling and analysis of our study problem. The Graphic User Interface (GUI) of GENOTECH is simple and user-friendly. The GRN is easily created as a directed graph using GUI. Given the correct set of logical parameters, it generates a state graph as an output which includes all possible steady states, stable states and their respective trajectories. Thus, the dynamical behaviour of a GRN is captured in the form of homeostasis (cycles) and epidgenetics(stable states).

3.2 Computation Tree logic (CTL)

The dynamics of a GRN intrinsically depend on the values of the logical parameters which are often not measurable in-vivo. Finding suitable classes of these parameters is a major problem in modeling of a GRN. Despite lack of precise data on these parameters, several qualitative biological observations and/or hypotheses regarding the behaviour of a GRN are detectable. For an example, homeostasis (respectively multistationarity) is an experimentally observable behavior and it indicates that a negative (respectively positive) circuit is functional [49] in a GRN. In order to derive the correct set of logical parameters for modeling a GRN, it is necessary to use formal methods in (model checking) with an ability to analyse the network structures. To this end, the use of Computation Tree Logic (CTL) was pioneered in [12] for the inference of logical parameters of a GRN. CTL is a type of model checking methodology which belongs to the family of branching-time logics, and is thus a natural choice for application on the discrete (qualitative) model of a GRN [20]. Given a set of biological properties (translated into respective CTL formulas), Model checking can verifing the properties a model checking that exist in the state graph (qualitative model) of the GRN.

3.2.1 Semantics of CTL

The formal semantics and specification of CTL grammar are described below. This section provides an overview of some common terminologies used in [12] for the analysis of a GRN.

Definition 3.7 (Atomic Proposition) *Each CTL sentence (also called CTL formula) in a GRN is an atomic proposition and stands for atomic facts which may hold in a system. Atomic propositions are of the form (a=n) where a is a variable of GRN and* $n \in Y_a$*);*Y_a *is a level of concentration of variabe "a" in a GRN.*

Several connectives and operators are used in conjunction with these atomic propositions. The usual connectives are $\neg$, $\wedge$, $\vee$, $\Rightarrow$ which denote not, and, or and implication respectively. Two types of CTL operators exist in CTL grammar (described in section 3.2 and 3.3).

Example of atomic proposition: As an example, (a=1) means that the concentration level of an entity *"a"* is 1 in the current state of a state graph of a GRN.

3.2.2 Path Quantifiers

Two path quantifiers exist in CTL grammar:

1. **A. 'A'** or $\forall$ (interpreted as for all) is a path quantifier which checks the existence of a certain property ϕ in all paths which start from a given state.

2. **E. 'E'** , or $\exists$ (interpreted as there exist) is the second path quantifier. It checks the existence of a certain property ϕ in at least one path starting from a given state.

3.2.3 State Quantifiers

1. **G** . *'G'* or $\Box$, is a state quantifier interpreted as globally. It refers all the states of a the entire subsequent path.

2. **F**. *'F'* or $\Diamond$ (it interpreted as ultimately) is another path quantifier which refers a state in a path.

3. X. X is a another state quantifier which refers to existence of a property ϕ in the immediately next state from a given state.

Each CTL operator is a pair of symbols in which the first one is a path quantifier and the second one is a state quantifier. Different cases of application of CTL operators are shown in Figure: 3.5, 3.6, 3.7 and 3.8 as examples.

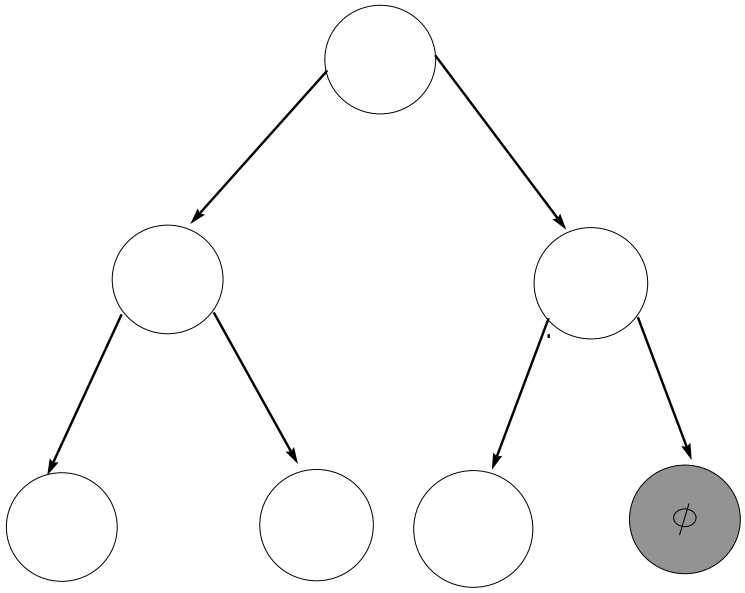


Figure 3.5: **EF** ϕ : An example showing the existence of a single computational path out of four total paths along which eventually the property ϕ holds.

In the case of our running example, the interaction graph depicting the mucus production system in P. aeruginosa (Figure 3.2) comprises of a positive and a negative feedback circuit. From a biological point of view, this makes possible a dynamic with two stationary states for some values of the parameters; an epigenetic change (from the non-mucoid state to the mucoid one) and a homeostatic change (from the non-mucoid state to non-mucoid state).

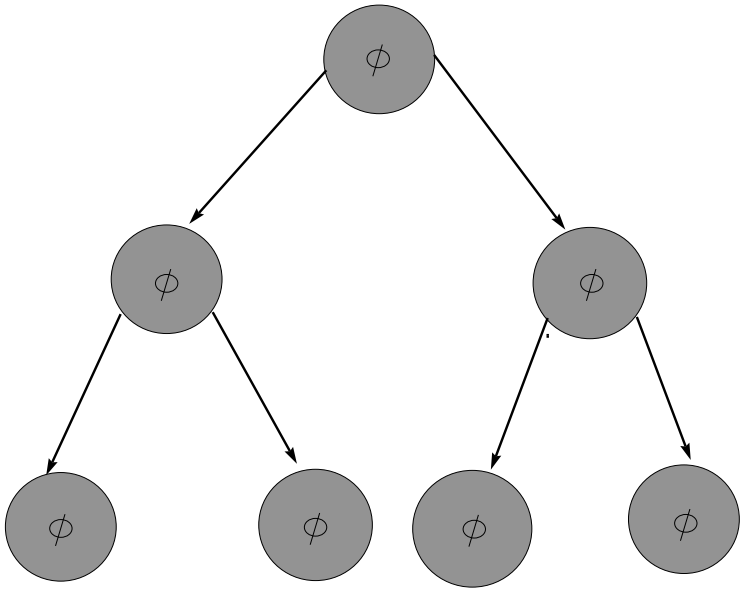


Figure 3.6: **AG** ϕ : An example of existence of a property ϕ along all the states of paths.

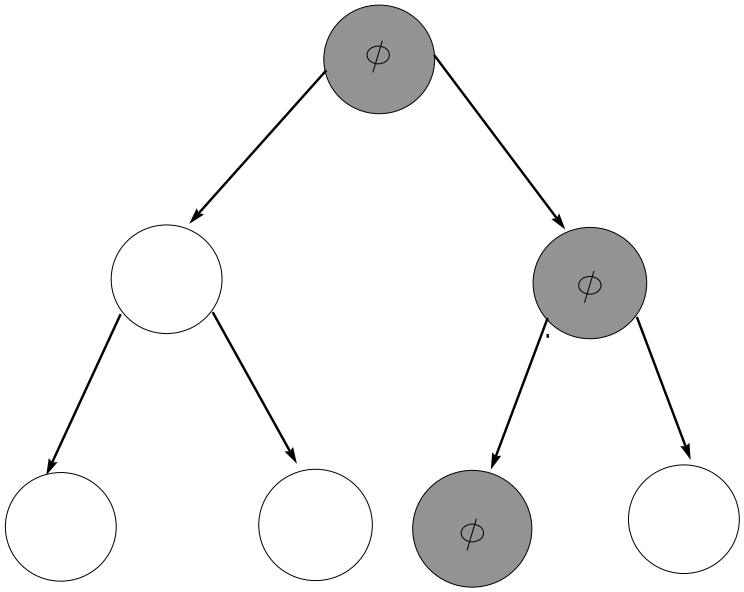


Figure 3.7: **EG** ϕ: An example of exixtence of a computational path along which a property ϕ always hold.

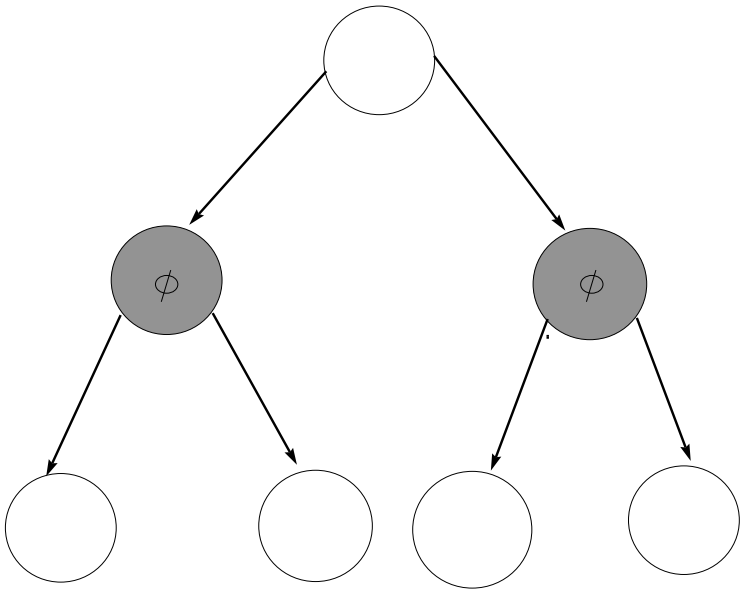


Figure 3.8: **AX** ϕ : An example of exixtence of a property ϕ in the successor state along all paths.

As an example, we discuss the case of the dynamical epigenetic hypothesis for P.aurignosa GRN (shown in Figure 3.2) tested through CTL formalism in [2]. The dynamical hypothesis of epigenetics logically translated into a CTL specification is expressed as:

- (a = 2) ⇒ AXAF (a = 2)

- (a = 0)⇒ AG (¬(a = 2))

The above statements imply that, if var. a (entity a in Pseudomonas aeruginosa GRN shown in Figure 3.2) is equal to 2 (qualitative level of concentration), then it will finally be equal to 2 (concentration) in the future and that if var. a is equal to 0 (concentration), then it will never be equal to 2 (concentration) in the future. Eight models satisfying the epigenetic hypothesis were retrieved through intensive model checking approach applied

in the software Selection of Models of Biological Networks (SMBioNet) [2]. This proved formally that the epigenetic hypothesis is consistent. The kinetic parameters of the GRN of figure 3.2 were deduced using this approach. We also deployed SMBioNet [2] to infer the logical parameters of our GRN (discussed in Chapter 4.) .

3.3 Hybrid Modeling

Hybrid models permit the integrated representation of both discrete and continuous features of a system. They have been widely and effectively utilized for the verification of real time and embedded systems. The hybrid modeling approach introduced in [2] for analysis of GRNs, combine the discrete modeling domain of Rene Thomas (discussed in section 3.1.1.) with continuous time domain (differential equation) in a single formalism. In this hybrid modeling formalism, a sigmoid-curve is converted into a piece-wise linear curve instead of a discretized curve (as shown in Figure 3.9). The delays required for gene evolution are represented in terms of time intervals and clocks. A clock represents a set of continuous variables. Each entity say "a" in a GRN is associated with a clock variable 'h_a' of gene "a" which evolves synchronously. The time measured by the clock variable 'h_a' between two discrete levels (of Thomas formalism) is called the delay between these levels. These clock intervals (delays) reflect the characteristics of continuous dynamics within the discrete formalism. Hybrid modeling is a powerful formalism for studying temporal properties i.e. time delays, which help in describing the system evolution associated with discrete dynamics (homeostasis, multistationarity). This approach has advantage in terms of saving computational time when compared to continuous approach. The formal semantics of this framework also known as Bio-LHA is adapted from [6],[2],[7] and is described below:

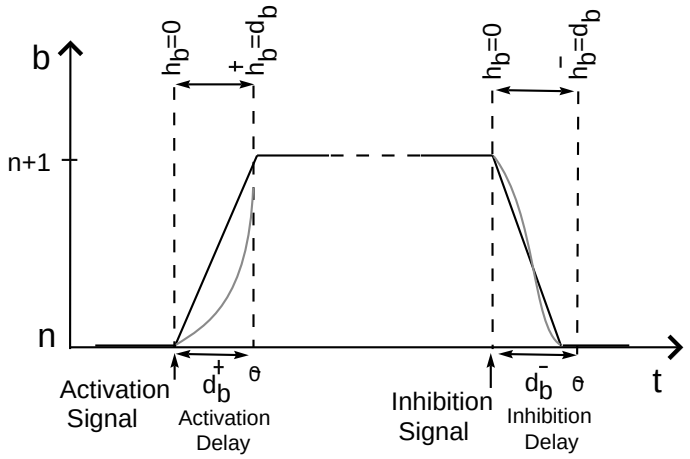


Figure 3.9: **Piece-wise linear representation of the activation and degradation:** In piece-wise linear dynamics, each entity is assigned a clock variable (h_b). Where, (h_b) represent the time elapsed from signal initiation until the value of delay parameter (Θ) is acquired.

3.3.1 Parametric Bio-LHA

Parametric Bio-LHA was originally proposed by [3] for the linear hybrid modeling of GRN. This framework refines the discrete (qualitative) model by incorporating clocks and time delays in it. The resultant model is then suitable for the computation of exact conditions in the form of delay constraints for the existence of the behaviors of GRN. Given X and P as the sets of real valued variables and parameters respectively, let us consider $C^{=}(X, P)$ (resp. $C^{\leq}(X, P)$ and $C^{\geq}(X, P)$) to represent the set of constraints using only $=$ (resp.$\leq$, $\geq$).

Definition 3.8 (Parametric Linear Hybrid Automaton Bio-LHA) *"In a parametric Bio-LHA, H represents a tuple ⟨Loc, l_0, X, P, E, Inv, Dif⟩ where:*

- *Loc represents a finite set of locations,*
- *$l_0 \in L_{0c}$ represents the initial location,*

- *P represents a finite set of parameters (delays)*
- *X represents a finite set of real-valued variables (clocks).*
- *E⊆ Loc* $\times$ $C^{=}(X, P)$ $\times 2^{X}\times$ *L is a finite set of edges, e=(l, g, R, l′) ∈E represents an edge from the location l to the location l′ with the guard g and the reset set R⊆X; we require that the set of clocks in g is a subset of R.*
- *Inv : Loc →* $C^{\leq}(X, P) \cup C^{\geq}$ *(X, P) assigns an invariant to any location.*
- *Dif : Loc × X→{0, 1, -1} maps each pair (l, h) to an evolution rate.*

  The semantics of a parametric Bio-LHA is a timed transition system. We define the semantics according to the time domain T. We let T=T/{0}".*

Definition 3.9 (Sementics of Bio-LHA) *"Let γ be a valuation for the parameters P and v represents the values of clocks in a location. The (T, γ)-semantics of a parametric Bio-LHA H = (Loc,* l_0*, X, P, E, Inv, Dif) is defined as a timed transition system* S_H*=(S,* s_0*, T, →) where: (1) S= {(l, v) | l ∈ L and v | = Inv(l)}; (2)* s_0 *is the initial state and (3) the relation →⊆S ×T×S is defined for t ∈ T as:*

- ***Discrete Transitions:***$(l, v) \xrightarrow{0} (l', v')$ *iff* $\exists(l, g, R, l') \in E$ *such that g(v)=true,* $v'(h) = 0$ *if* $h \in R$ *and v'*$(h) = v(h)$ *if* $h \not\in R$*.*
- ***Continuous Transitions:*** *For* $t \in T*, (l, v) \longrightarrow (l', v')$ *iff* $l' = l, v'(h) = v(h) + Dif(l, h) \times t$*, and for every* $t' \in [0, t], (v(h) + Dif(l, h) \times t') \models Inv(l)$*."*

The Bio-LHA of our running example is shown in Figure 3.10. Analysis of such hybrid models using HyTech reveals how the clocks and delays map the continuous evolution from a discrete model by taking into account the temporal behaviour in the expression space.

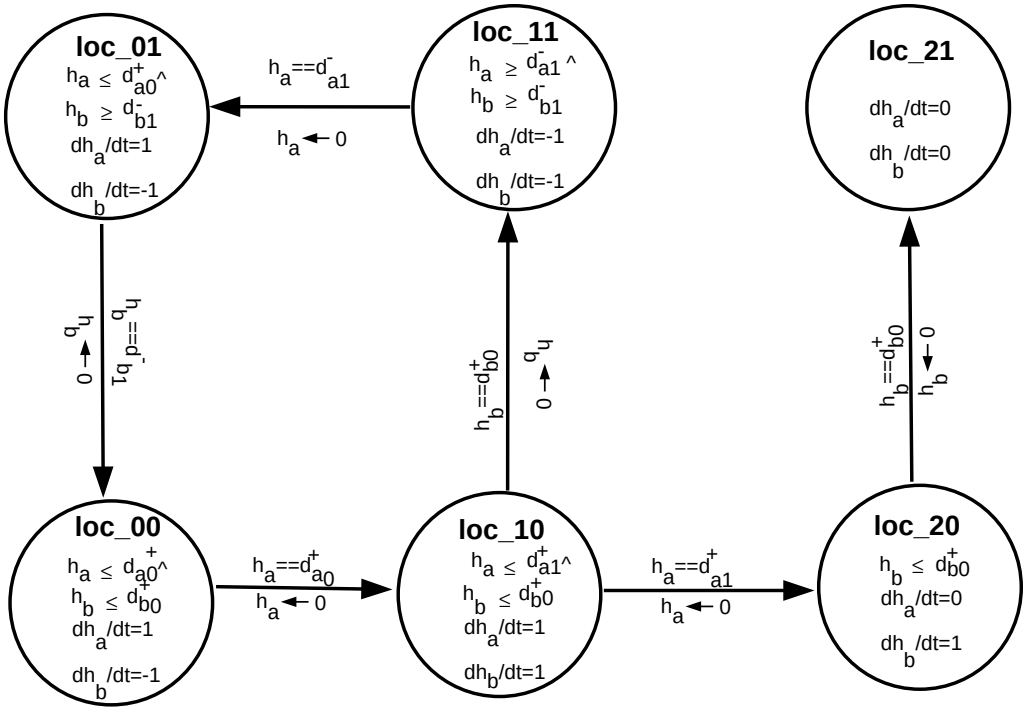


Figure 3.10: **Bio-LHA of P.aeruginosa (as shown in 3.3):** Circles represent the locations and arrows represent discrete transitions from one location to another. h_a, h_b, and d_a, d_b represent the clock variables and delays for entities *"a"* and *"b"* respectively.

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3.3.2 Temporal Domain

Based on the framework and semantics defined above, the Bio-LHA represents a hybrid model where temporal domain superposes the expression space. Several properties of temporal domain according to this framework [2] are adapted and include:

- **Temporal Zone:** *A temporal zone is a region where time elapses until the discrete change of expression level of one variable occur.*

This means that a temporal zone refers to the continuous transition in which the time elapses such that the location remains the same along with the satisfaction of location invariants. An example of a temporal zone is shown in Figure 3.11

- **Temporal State Space:** *Union of all temporal zones of the system refers to the temporal state space (hybrid state space).*

  This temporal state space is formed by union of expression state space (discrete states) and temporal domain (temporal zones). The approach of [2] allows to analyze the dynamics of temporal state space by synthesizing the delay constraints of the trajectories leading to Invariance Kernel (described below) and the stable steady state.

- **Invariance Kernel (IK):** *A large set of cyclic trajectories in temporal state space which start and end at the same points.*

  IK gives information about the behavior of the cycle. If the system bifurcates from the IK then it will move towards divergence trajectories leading to stable steady states.

The hybrid modeling of P. aeruginosa mucus production system was analyzed using Hytech [29], [30] which is a software for automatic analysis of hybrid systems. The temporal state space of P. aeruginosa along with the invariant trajectory analyzed through Hytech is shown below (Figure 3.12).

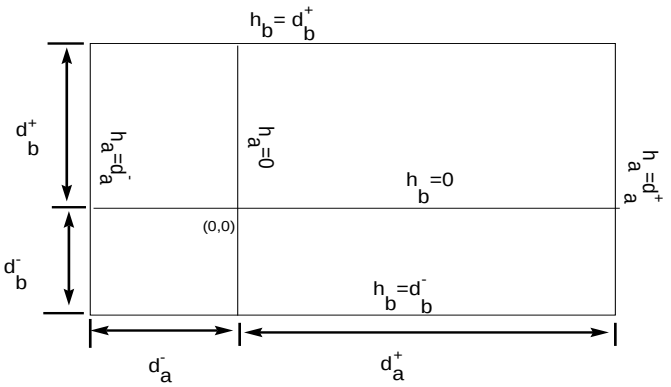

Figure 3.11: **Temporal zone:** An example of a temporal zone corresponding to the state (0,0)of the qualitative model of Pseudomonas aeruginosa in Figure 3.4

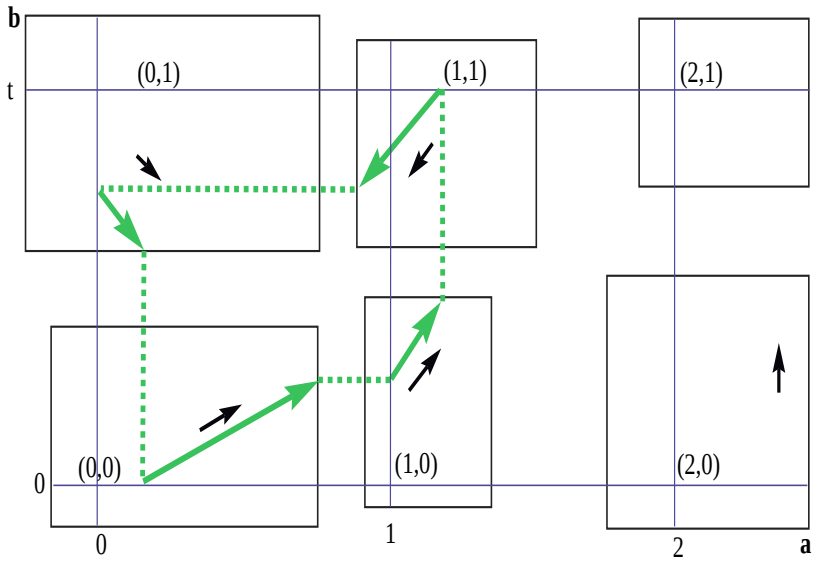

Figure 3.12: **Temporal State Space:** Complete state space of temporal zones of the discrete model of Pseudomonas aeruginosa(Figure:3.2) along with the speed direction of clocks is shown. Invariant trajectory of Pseudomonas aeruginosa derived from the hybrid modeling is shown with arrows in green colour

Chapter 4

Results and Discussion

This chapter presents the modeling and analysis results of p53-Akt associated GRN (explained in chapter 2) using the methodology and software tools explained in the previous chapter. First subsection (4.1) describes the reults obtained using discrete modeling of p53 and Akt associated GRN and second subsection (4.2) describes the results obtained using hybrid modeling of p53 and Akt associated GRN.

4.1 Qualitative Results

4.1.1 Logical Parameters

The logical parameters of GRN (shown in Figure 4.1) were obtained using SMBioNet. Source file of SMBioNet is given in appendix A . The conjuncted CTL formulas used to infer these parameters are as follows:

$$Init \rightarrow \forall X\ (\exists F\ Init) \wedge$$

$$Init \rightarrow \exists F\ (Akt{=}1) \rightarrow Init \wedge$$

$$Init \rightarrow \exists F(\exists G(p53{=}0 \wedge Mdm2c{=}1 \wedge Mdm2n{=}1 \wedge Akt{=}1))$$

The initial state *Init* in the CTL formulas, represents the state where all entities are at level 0 (i.e., $Init \rightarrow p53{=}0 \wedge Mdm2n{=}0 \wedge Akt{=}0 \wedge Mdm2c{=}0$).

The first CTL formula is used to enforce the physiological condition where all entities evolve to maintain homoeostatis. This CTL formula checks the existence of cyclic trajectories (a path leading and ending into the same given state) from an initial state in the absence of any perturbation (no stress).

The next formula encodes the physiological behaviour of a cell in case of a stress signal. In such cases, usually the cell goes from an initial state to a stress state and then comes back to the initial state. This stress state refers to an intermediate state where cell takes time to repair the stress using naturally existing self defense mechanism. However, if the stress pursues constitutively, the cell usually proceeds to a diseased state causing harmful effects in the body. To identify the trajectories which could lead to the diseased state starting from a normal state (Init), third CTL formula was used.

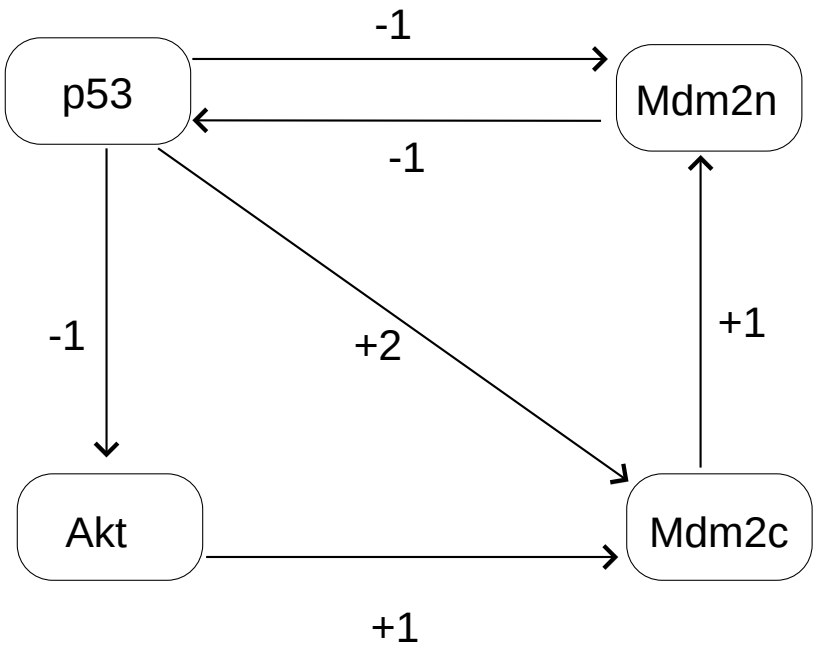

Figure 4.1: **The p53-Akt associated Gene Regulatory Network (GRN).** Figure shows the entities and transitions involved in the regulation of p53 (a tumor supressor gene). p53 is regulated via three feedback loops: A negative feedback loop which involves Mdm2n and a positive feedback loop which involves Akt,Mdm2c and Mdm2n.

SMBioNet generated six hundred and forty eight parameters sets, out of which only five fulfilled the described CTL formula (Table 4.1). Each of these parameters sets were further analysed which highlighted the differences in three parameters only. Final selection of the parameters set was based on already reported data [1] and is described below:

1. The parameter $K_{p53(Mdm2n)}$ shows that the only regulator and inhibitor Mdm2n of p53 is absent. Therefore, it was assumed that the activity of p53 would achieve its maximum level [1]. Thus the value '2' was selected for this parameter, leaving three parameter sets to choose from.

2. The parameter $K_{Mdm2c(p53)}$ shows that in the presence of p53 (which is an activator of Mdm2c), Mdm2c would be activated [1]. Thus the value '1' (active) was selected for this parameter, leaving two parameter sets to choose from.

3. The parameter $K_{Mdm2c(p53,Akt)}$ shows that in the presence of p53 & Akt (activators of Mdm2c), Mdm2c would be activated. Out of the remaining two parameter sets only one showed the value '1' (active) and was selected.

S.no	K_Parameters	Availaible Resources	MODEL #1	MODEL #2	MODEL #3	MODEL #4	MODEL #5
1	K_{p53}	ϕ	0	0	0	0	0
		Mdm2n	1	2	1	2	2
2	K_{Akt}	ϕ	0	0	0	0	0
		p53	1	1	1	1	1
3	K_{Mdm2c}	ϕ	0	0	0	0	0
		p53	0	0	1	1	1
		Akt	1	1	1	1	1
		p53, Akt	1	1	1	1	1
4	K_{Mdm2n}	ϕ	0	0	0	0	0
		p53	0	0	0	0	0
		Mdm2c	1	1	1	1	1
		p53,Mdm2c	1	1	1	1	1

Table 4.1: **Logical parameters of p53 -Akt associated GRN.** The table lists the logical parameters of each model (5/648) which satisfied the CTL formulas. The parameters set of model number four was selected.

4.1.2 Discrete Model

The selected parameters set of model 4 in Table 4.1 was used to model the p53-Akt associated GRN in GENOTECH. The resultant state graph (discrete model) as shown in Figure 4.2 consisted of 79 cycles among 23 states and a deadlock state (diseased state). This deadlock state represents the condition where the system could converge in case of any perturbation (stress response). Thus, the discrete model depicts the existence of homeostatic and epigenetic behavior in the p53-Akt associated GRN. The state (0,0,0,0) represents the virgin state in the discrete model (Figure 4.2). This is the initial state of the system without any perturbation. The discrete model was further analyzed to identify the trajectories (paths) starting from the initial state leading to the normal or diseased behavior respectively.

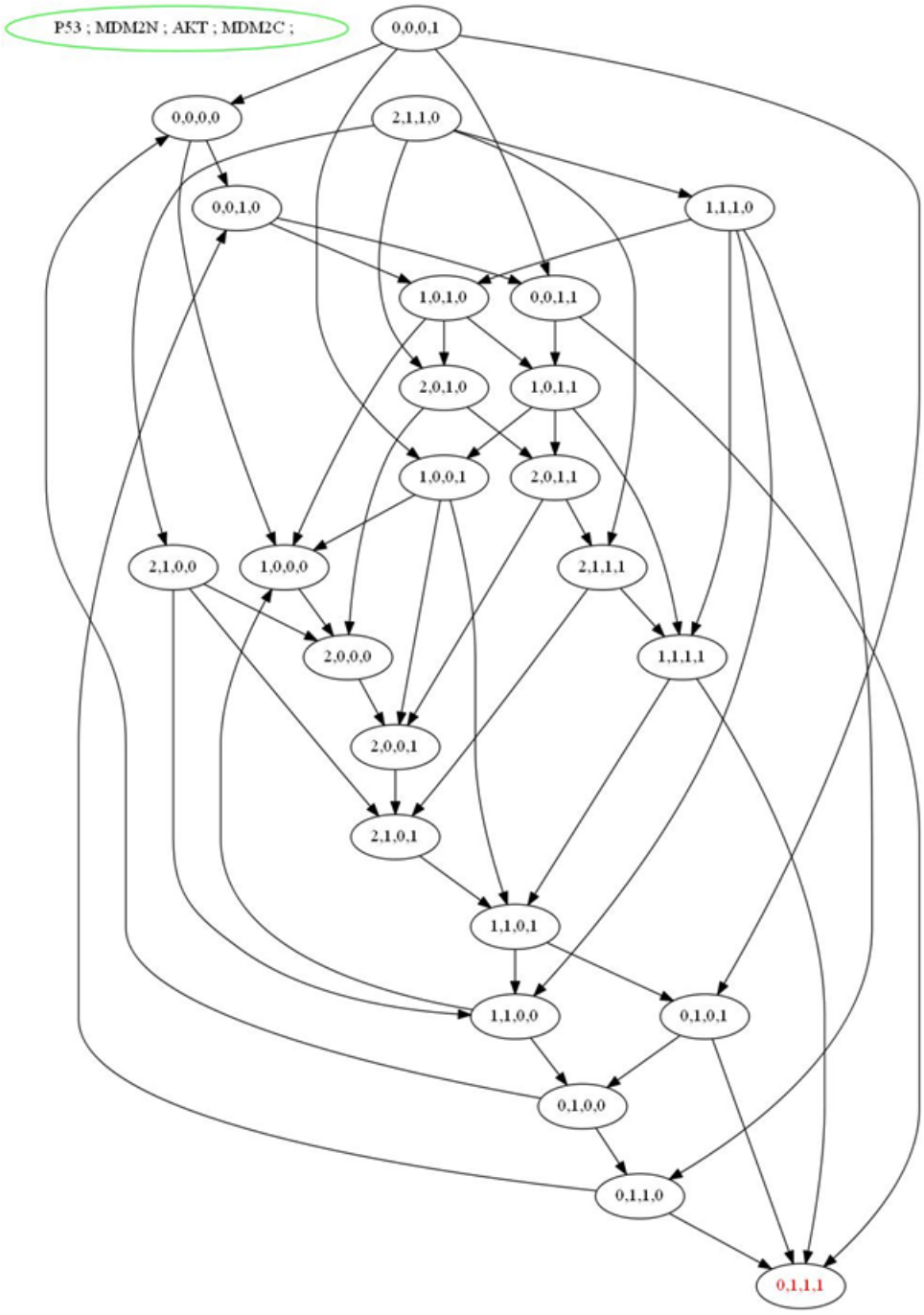

Figure 4.2: **Discrete model of the p53-Akt associated GRN.** The discrete model comprises of twenty four states and fifty two transitions. It contains seventy nine cycles and a deadlock state (0111).

The analysis of the discrete model revealed the existence of oscillation of each entity in only ten cycles (10/79). The ten cycles are shown in Table 4.2. All other cycles thus, depict redundancy in terms of evolution of entities and were not taken further. Further analysis of the ten cycles (Table 4.2) helped us to narrow down the analysis to only one cycle (Row number two in the Table 4.2) which showed oscillatory behavior in the correct physiological order associated with p53-Akt associated GRN. This cycle is shown in Figure 4.3.

Cycle $n°1$	[0, 0, 1, 0]→ [1, 0, 1, 0]→ [2, 0, 1, 0] → [2, 0, 1, 1] → [2, 1, 1, 1] → [2, 1, 0, 1] → [1, 1, 0, 1] → [0, 1, 0, 1] → [0, 1, 0, 0] → [0, 0, 0, 0] → [0, 0, 1, 0]
Cycle $n°2$	[[0, 0, 1, 0] → [1, 0, 1, 0] → [2, 0, 1, 0] → [2, 0, 1, 1] → [2, 1, 1, 1] → [2, 1, 0, 1] → [1, 1, 0, 1] → [1, 1, 0, 0] → [0, 1, 0, 0] → [0, 0, 0, 0] → [0, 0, 1, 0]]
Cycle $n°$ 3	[[0, 0, 1, 0] → [1, 0, 1, 0] → [2, 0, 1, 0] → [2, 0, 1, 1] → [2, 0, 0, 1] → [2, 1, 0, 1] → [1, 1, 0, 1] → [0, 1, 0, 1] → [0, 1, 0, 0] → [0, 0, 0, 0] → [0, 0, 1, 0]]
Cycle $n°4$	[[0, 0, 1, 0] → [1, 0, 1, 0] → [2, 0, 1, 0] → [2, 0, 1, 1] → [2, 0, 0, 1] → [2, 1, 0, 1] → [1, 1, 0, 1] → [1, 1, 0, 0] → [0, 1, 0, 0] → [0, 0, 0, 0] → [0, 0, 1, 0]]
Cycle $n°5$	[[0, 0, 1, 0] → [1, 0, 1, 0] → [1, 0, 1, 1] → [2, 0, 1, 1] → [2, 1, 1, 1] → [1, 1, 1, 1] → [1, 1, 0, 1] → [0, 1, 0, 1] → [0, 1, 0, 0] → [0, 0, 0, 0] → [0, 0, 1, 0]]
Cycle $n°6$	[[0, 0, 1, 0] → [1, 0, 1, 0] → [1, 0, 1, 1] → [2, 0, 1, 1] → [2, 1, 1, 1] → [1, 1, 1, 1] → [1, 1, 0, 1] → [1, 1, 0, 0] → [0, 1, 0, 0] → [0, 0, 0, 0] → [0, 0, 1, 0]]
Cycle $n°7$	[[0, 0, 1, 0] → [1, 0, 1, 0] → [1, 0, 1, 1] → [2, 0, 1, 1] → [2, 1, 1, 1] → [2, 1, 0, 1] → [1, 1, 0, 1] → [0, 1, 0, 1] → [0, 1, 0, 0] → [0, 0, 0, 0] → [0, 0, 1, 0]]
Cycle $n°8$	[[0, 0, 1, 0] → [1, 0, 1, 0] → [1, 0, 1, 1] → [2, 0, 1, 1] → [2, 1, 1, 1] → [2, 1, 0, 1] → [1, 1, 0, 1] → [1, 1, 0, 0] → [0, 1, 0, 0] → [0, 0, 0, 0] → [0, 0, 1, 0]]
Cycle $n°9$	[[0, 0, 1, 0] → [1, 0, 1, 0] → [1, 0, 1, 1] → [2, 0, 1, 1] → [2, 0, 0, 1] → [2, 1, 0, 1]→ [1, 1, 0, 1] → [1, 1, 0, 0]→ [0, 1, 0, 0] → [0, 0, 0, 0] → [0, 0, 1, 0]]
Cycle $n°10$	[[0, 0, 1, 0] → [1, 0, 1, 0] → [1, 0, 1, 1]→ [2, 0, 1, 1] → [2, 0, 0, 1] → [2, 1, 0, 1] → [1, 1, 0, 1] → [1, 1, 0, 0] → [0, 1, 0, 0] → [0, 0, 0, 0] → [0, 0, 1, 0]]

Table 4.2: **Cyclic trajectories of discrete model (Figure 4.2) exhibiting oscillatory behavior**. The table shows state transitions of each of the unique cyclic trajectory.

The order of configuration (state) in the cycle (Figure 4.3) is (entity order: p53,Mdm2n,Akt,Mdm2c). Initially the system is in a virgin state (0,0,0,0) where all entities are at level 0. In case of a stress signal a new state is obtained i.e., (0,0,1,0), when akt is activated. Akt in turn activates

p53 causing a shift to a new state (1,0,1,0). p53 at its level 2 causes the activation of Mdm2c and Mdm2n and the respective states become (2,0,1,1) and (2,1,1,1). Mdm2n causes inhibitory effect on Akt by down regulating it and the system state is shifted to (2,1,0,1). Mdm2c further increases the expression of Mdm2n and recovers the whole stress condition by following respective state shifts: (2,1,0,1) → (1101)→ (1100) →(0100) →(0000).

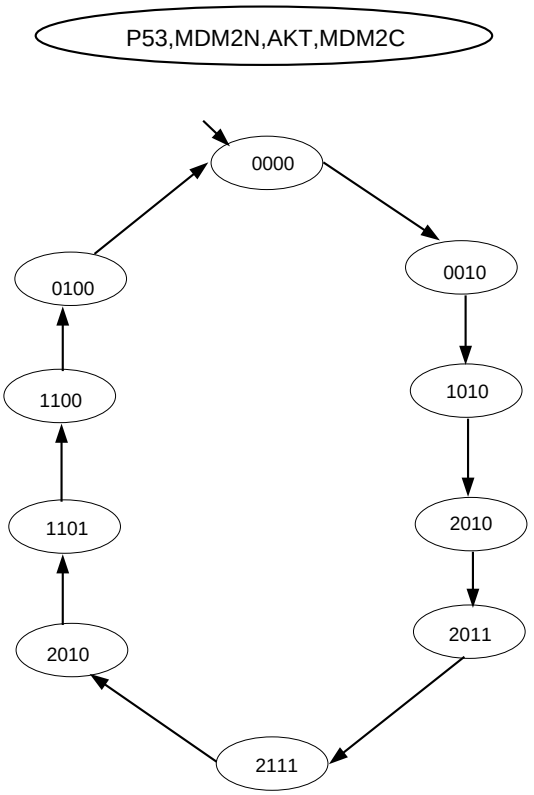

Figure 4.3: **Homoeostatic cycle of p53-Akt associated GRN.** The cycle shows the normal functioning in un-perturbed condition.

All divergent trajectories, from the virgin state leading to the diseased state, are shown in Figure 4.6 .The transitions leaving the cyclic trajectory and leading to the divergent (towards (0,1,1,1) in this case) trajectory are called bifurcation points. There exists four states ((0,0,1, 0),(1,0,1,0), (1,1,0,1), (0,1,0,0)) in the cyclic trajectory that bifurcates to the divergent

trajectory (Figure 4.6. However, each of these divergent trajectories also contains at least one path which leads back into the cyclic trajectory.

- State (0,0,1,0) leads three paths towards the diseased state. Only Akt is active in this state. The transition (0,0,1,0) $\rightarrow$ (1,0,1,0) maintains the cyclic behavior, whereas the transition (0,0,1,0) $\rightarrow$ (0,0,1,1) exits the cycle from this state.

- In state (0,1,0,0), the level of nuclear Mdm2 is kept at basal level in order to keep the p53 under control. The divergent trajectory follows (0,1,0,0) $\rightarrow$ (0,1,1,0) transition to exit the cyclic trajectory from this state (shown in Figure 4.6).

- In state (1,1,0,1) p53 is present and Akt is absent. Bifurcation point from this state is (1,1,0,1) $\rightarrow$ (0,1,0,1) (shown in Figure 4.6).

- The state (1,0,1,0) shows the presence of both Akt and p53 and exits the cyclic trajectory through (1,0,1,0) $\rightarrow$ (1,0,1,1) (shown in Figure 4.6).

Figure 4.4 and 4.5 represent two more cycles from the state graph which have three and four bifurcation points respectively. These cycles have higher probability for leading to a divergent trajectory (towards (0,1,1,1)). In these cycles (shown in Figure 4.5 and 4.6) it can be noted that Akt is activated in more states than p53. This may be one reason for more bifurcation states in these cycles. All other cycles listed in Table 4.2, p52 is activated in more states than Akt and thus have zero, one or two bifurcation states.

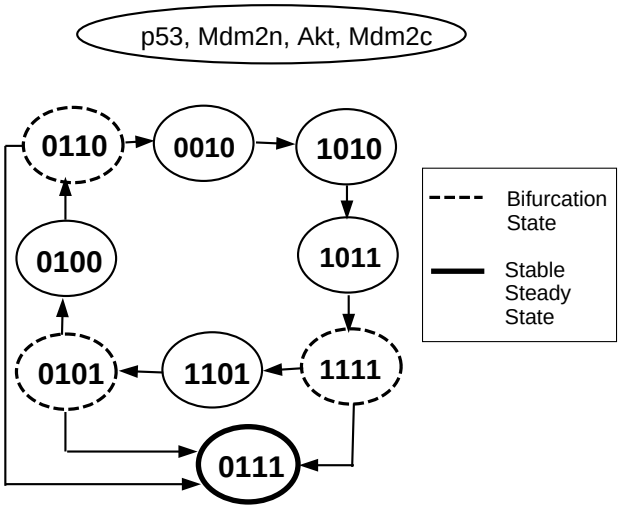

Figure 4.4: **A cycle of p53-Akt associated GRN.** The cycle show three bifurcation states leading towards the divergent trajectory.

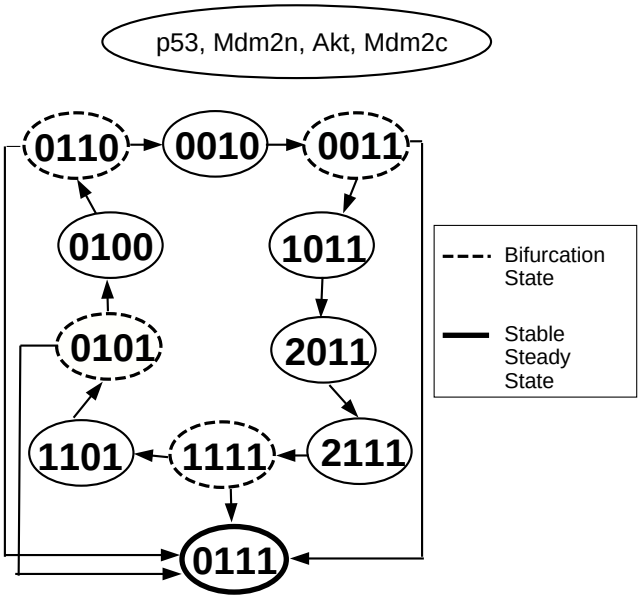

Figure 4.5: **A cycle of p53-Akt associated GRN**. The cycle shows four bifurcation states leading towards the divergent trajectory.

Further analysis through hybrid model provided the delay constraints (re-

spectively the biological conditions) required for the viability of homeostatic behavior. Any deviation from these constraints could lead the system to converge to stable steady state (discussed in section 4.2).

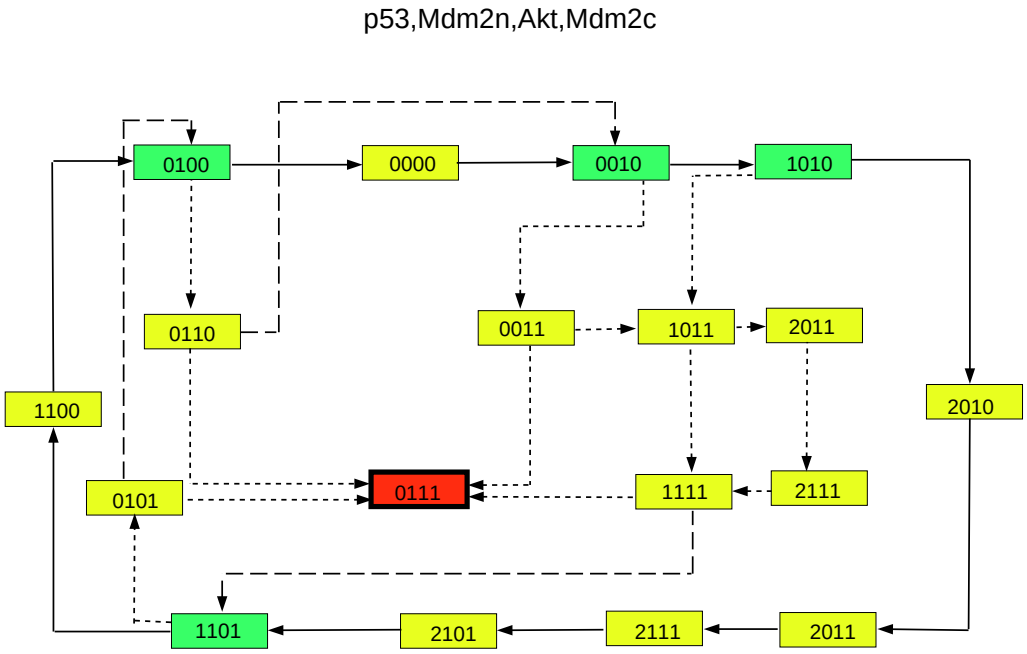

Figure 4.6: **Normal and divergent trajectories.** Each box represents a unique state(configuration). The values 0,1,2 inside the box represent qualitative levels of entities in the order (p53, Mdm2n, Akt, Mdm2c). Solid lines indicate transitions involved in cyclic trajectory. Small dashed lines indicate the transitions involved in the divergent trajectory. Long dashed lines indicate the transitions of the divergent trajectory that leads back to cyclic trajectory. The box with bold line indicates the stable steady state (0,1,1,1).

4.2 HyTech Results

4.2.1 Linear Constraints

In this section, the results of hybrid modeling are discussed. The cycle selected from the discrete model (shown in figure 4.3) was converted into a BIO-LHA using the methodology explained in Section 3.3.1. The source

code used for the conversion of discrete model into the hybrid model is given in appendix B.

The delay constraints of the cycle generated using Hytech are given below (Table 4.3). These constraints (delay parameters) actually state the biological conditions which should be satisfied to maintain this cyclic behavior. Any deviations from these conditions could lead to the divergent trajectory towords (0,1,1,1). In table 4.3 d^+ and d^- refer to the activation (positive) and inhibition (negative) delays respectively.

S.No	Delay Constraints
1	$d^+_{AKT} <= \mid d^-_{P53} \mid + d^+_{P53}$
2	$2d^+_{P53} <= d^+_{MDM2c}$
3	$d^+_{MDM2n} <= \mid d^-_{AKT} \mid$
4	$d^+_{AKT} + \mid d^-_{AKT} \mid < = \mid 2d^-_{P53} \mid + d^+_{MDM2c}$
5	$d^+_{AKT} + \mid d^-_{MDM2c} \mid + \mid d^-_{AKT} \mid < = \mid 3d^-_{P53} \mid + d^+_{MDM2c}$
6	$d^+_{MDM2c} + \mid d^-_{P53} \mid <= d^+_{MDM2n} + d^+_{AKT}$
7	$d^+_{MDM2c} + 2\mid d^-_{P53} \mid <= d^+_{AKT} + \mid d^-_{AKT} \mid + \mid d^-_{MDM2c} \mid$
8	$\mid d^-_{P53} \mid <= \mid d^-_{MDM2n} \mid$
9	$d^+_{AKT} >= \mid d^-_{MDM2n} \mid$

Table 4.3: **Delay constraints of homoeostatic cycle.** Table lists all the delay constraints of the cyclic trajectory shown in Figure 4.3 using hyTech.

The constraints shown in Table 4.3 represents the conditions for the existence of homoeostasis. If the values of the delays do not satisfy these constraints then the oscillation will no more exist. Constraint 1 (in Table 4.3) shows that the time delay for activation of Akt is less than or equal to the sum of both the activation and inhibition time delays of p53. This constraint also support our prediction about the role of p53 and Akt in apoptosis and anti-apoptosis. This means that p53 completes its period slowly than the activation of Akt. This is a new prediction as compared to the predictions of [1]. Constraint 2 represents that the positive delay of p53 is less than or equal to th positive delay of Mdm2c. Other constraints can be interpreted similarly.

4.3 Limitations

The use of SMBioNet and HyTech for modeling and analysis faces state explosion problem and poses a restriction to the number of variables being used. Both of them can cater small networks with number of variables typically less than 7 [41]. However, this problem can be solved by using parallel programming.

Chapter 5

Conclusion

p53 and Akt are very important proteins acting in harmony to control the proliferation of cells under normal physiological conditions. Their biological behavior is best described as an antagonist switch which keeps the cell in either a pro-survival state (low p53 and high Akt levels) or a pro-apoptotic state (high p53 and low Akt levels). Altered protein expressions of both p53 and Akt have been observed in a number of human tumors [24]. However the dynamics of this antagonistic behavior leading to the tumor progression has remained unclear so far. The present study conducted an exploratory analysis to study the dynamics of p53-Akt associated GRN (discussed in Chapter 1). The study presented a formal model and its subsequent analysis for elucidating the dynamics of p53-Akt associated GRN, which would be cost consuming and time taking if analyse in wetlab. The formal model was proposed and analyzed using several approaches like model checking, kinetic logic and hybrid modeling.

Initially, the GRN comprising of the entities p53, Akt and Mdm2c and Mdm2n was modeled using kinetic logic of René Thomas. The logical parameters for the qualitative model were infered using the model checking approach implemented in SMBioNet. The Qualitative model was further enriched with time delays as unvalued parameters and then this refined hybrid model was analyzed for the unvalued delay constraints representing the existence of the qualitative cycles.

The results of our study (Section 4.1 and 4.2) revealed many interesting features involved in the existence of such tumor promoting behavior mediated by p53 and Akt. The analysis of qualitative model of p53-Akt associated GRN predicted the existence of one stable state (0,1,1,1) and several cycles representing epigenetic and homeostatic behavior of the cell respectively. The discrete model (Figure 4.2) of p53-Akt associated GRN revealed the same oscillatory behaviour of p53 as discussed in [1], under basal conditions. Moreover, the addition of Akt mediated positive feedback loop in our model revealed different trajectories and configuration which leads to the deadlock state (0,1,1,1). This deadlock state actually depicts the tumor favoring state. The low level of p53 along with high levels of Akt favours the cancer (tumor) in two ways:

1. The constitutive low level of p53 promotes the cellular repair process to halt and does not work despite of damage/stress. Therefore the cell with mutated DNA also commits for replication process.

2. The constitutive Akt levels gives the proliferative advantage to both normal/damaged cells.

The exact trajectories leading to the tumor favoring state (0,1,1,1) are also predicted. Moreover the delay parameters (biological conditions) of the homeostatic cycle of p53-Akt associated GRN were synthesized. These delay parameters represent the viability conditions for the cell to exhibit normal function.

The presented model clearly depicts the significance of Akt mediated regulation of p53 by revealing the dynamics involved in the network.

5.1 Future Work

The present study can be extended in two dimensions:

1. The computational analysis of p53-Akt associated network GRN can be further extended to analyse other features of invariance kernel like

volume, diameter etc. This could help in analyzing the stability of invariance kernel. Furthermore, these results can be used for quantitative modeling and analysis which could aid in modeling of specialized conditions equivalent to in-vivo knock-out experimentations.

2. The p53-Akt associated GRN could be extended to include more proteins which are involved in the progression of cancer. This could aid in the identification of biomarkers and/or therapeutic targets involved in different types of cancers.

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Appendix A

SMBioNet Source File

VAR
p53 = 0 2 ;
MDM2n = 0 1 ;
MDM2c = 0 1 ;
AKT = 0 1 ;
REG
MDM2n [(MDM2n¡1)]=>p53 ;
p53 [(p53¡1)]=>MDM2n ;
MDM2c [(MDM2c>=1)]=>MDM2n ;
p53 [(p53>=2)]=>MDM2c ;
AKT [(AKT>=1)]=>MDM2c ;
p53 [(p53¡1)]=>AKT ;
PARA
Parameters for p53
K_{p53} = 0 2 ;
$K_{p53+MDM2n}$ = 0 2 ;
Parameters for MDM2n
K_{MDM2n} = 0 1 ;
$K_{MDM2n+MDM2c}$ = 0 1 ;
$K_{MDM2n+p53}$ = 0 1 ;
$K_{MDM2n+MDM2c+p53}$ = 0 1 ;

Parameters for MDM2c

$K_{MDM2c} = 0\ 1$;

$K_{MDM2c+AKT} = 0\ 1$;

$K_{MDM2c+p53} = 0\ 1$;

$K_{MDM2c+AKT+p53} = 0\ 1$;

Parameters for AKT

$K_{AKT} = 0\ 1$;

$K_{AKT+p53} = 0\ 1$;

CTL

$((p53 = 0 \& MDM2c = 0 \& MDM2n = 0 \& AKT = 0) \rightarrow EF(AKT = 1) \rightarrow EF(p53 = 0 \& MDM2c = 0 \& MDM2n = 0 \& AKT = 0))$

&

$((p53 = 0 \& MDM2c = 0 \& MDM2n = 0 \& AKT = 0) \rightarrow EX(EF(p53 = 0 \& MDM2c = 0 \& MDM2n = 0 \& AKT = 0)))$

&

$((p53 = 0 \& MDM2c = 0 \& MDM2n = 0 \& AKT = 0) \rightarrow EF(EG(p53 = 0 \& MDM2c = 1 \& MDM2n = 1 \& AKT = 1)))$

 # MODEL 1

$K_{p53} = 0$

$K_{p53+MDM2n} = 1$

$K_{MDM2n} = 0$

$K_{MDM2n+MDM2c} = 1$

$K_{MDM2n+p53} = 0$

$K_{MDM2n+MDM2c+p53} = 1$

$K_{MDM2c} = 0$

$K_{MDM2c+AKT} = 1$

$K_{MDM2c+p53} = 0$

$K_{MDM2c+AKT+p53} = 1$

$K_{AKT} = 0$

$K_{AKT+p53} = 1$

 # MODEL 2

$K_{p53} = 0$

$K_{p53+MDM2n} = 2$

$K_{MDM2n} = 0$
$K_{MDM2n+MDM2c} = 1$
$K_{MDM2n+p53} = 0$
$K_{MDM2n+MDM2c+p53} = 1$
$K_{MDM2c} = 0$
$K_{MDM2c+AKT} = 1$
$K_{MDM2c+p53} = 0$
$K_{MDM2c+AKT+p53} = 1$
$K_{AKT} = 0$
$K_{AKT+p53} = 1$
MODEL 3
$K_{p53} = 0$
$K_{p53+MDM2n} = 1$
$K_{MDM2n} = 0$
$K_{MDM2n+MDM2c} = 1$
$K_{MDM2n+p53} = 0$
$K_{MDM2n+MDM2c+p53} = 1$
$K_{MDM2c} = 0$
$K_{MDM2c+AKT} = 1$
$K_{MDM2c+p53} = 1$
$K_{MDM2c+AKT+p53} = 1$
$K_{AKT} = 0$
$K_{AKT+p53} = 1$
MODEL 4
$K_{p53} = 0$
$K_{p53+MDM2n} = 2$
$K_{MDM2n} = 0$
$K_{MDM2n+MDM2c} = 1$
$K_{MDM2n+p53} = 0$
$K_{MDM2n+MDM2c+p53} = 1$
$K_{MDM2c} = 0$
$K_{MDM2c+AKT} = 1$
$K_{MDM2c+p53} = 1$

$K_{MDM2c+AKT+p53} = 1$
$K_{AKT} = 0$
$K_{AKT+p53} = 1$
MODEL 5
$K_{p53} = 0$
$K_{p53+MDM2n} = 2$
$K_{MDM2n} = 0$
$K_{MDM2n+MDM2c} = 1$
$K_{MDM2n+p53} = 0$
$K_{MDM2n+MDM2c+p53} = 1$
$K_{MDM2c} = 0$
$K_{MDM2c+AKT} = 1$
$K_{MDM2c+p53} = 1$
$K_{MDM2c+AKT+p53} = 1$
$K_{AKT} = 1$
$K_{AKT+p53} = 1$
SELECTED MODELS / CHECKED MODELS = 5 / 648 (5s924ms)

Appendix B

HyTech Code-Cycle 12

var

hP53,hMDM2N,hAKT,hMDM2C,h :analog;

k,n,l :discrete;

dpP53, dnP53, dpMDM2N, dnMDM2N, dpAKT, dnAKT, dpMDM2C, dnMDM2C :parameter;

automaton auto synclabs: ;

initially loc_0000;

– gĉne $n°0$ = P53

– gĉne $n°1$ = MDM2N

– gĉne $n°2$ = AKT

– gĉne $n°3$ = MDM2C

– pour la configuration 0,0,0,0

loc loc_0000:

while hAKT <= dpAKT wait dhP53 = 1, dhMDM2N = 0, dhAKT = 1, dhMDM2C = 1, dh = 1

when hAKT=dpAKT do hAKT'=0, k'=k+1, l'=l+h, h'=0 goto loc_0010;

– pour la configuration 0,0,1,0

loc loc_0010:

while hP53 <= dpP53 wait dhP53 = 1, dhMDM2N=1, dhAKT=-1, dhMDM2C=1, dh=1

when hP53=dpP53 do hP53'=0, k'=k+1, l'=l+h, h'=0 goto loc_1010;

– pour la configuration 0,1,0,0

loc loc_0100:

while hMDM2N >=dnMDM2N wait dhP53=1, dhMDM2N=-1, dhAKT=1, dhMDM2C=1, dh=1

when hMDM2N=dnMDM2N do hMDM2N'=0, k'=k+1, l'=l+h, h'=0 goto loc_0000;

– pour la configuration 1,0,1,0

loc loc_1010:

while hP53 <= dpP53 wait dhP53 = 1, dhMDM2N = 1, dhAKT =- 1, dhMDM2C = 1, dh = 1

when hP53 = dpP53 do hP53'=0, k'=k+1, l'=l+h, h'=0 goto loc_2010;

– pour la configuration 1,1,0,0

loc loc_1100:

while hP53 >= dnP53 wait dhP53 =- 1, dhMDM2N =- 1, dhAKT = 1, dhMDM2C = 0, dh = 1 when hP53=dnP53 do hP53'=0, k'=k+1, l'=l+h, h'=0 goto loc_0100;

– pour la configuration 1,1,0,1 loc loc_1101:

while hMDM2C>=dnMDM2C wait dhP53=-1,dhMDM2N=-1,dhAKT=1, dhMDM2C=-1, dh=1 when hMDM2C=dnMDM2C do hMDM2C'=0, k'=k+1, l'=l+h, h'=0 goto loc_1100;

– pour la configuration 2,0,1,0 loc loc_2010:

while hMDM2C <=dpMDM2C wait dhP53=0,dhMDM2N=1,dhAKT=-1,dhMDM2C=1,dh=1 when hMDM2C=dpMDM2C do hMDM2C'=0, k'=k+1, l'=l+h, h'=0 goto loc_2011;

– pour la configuration 2,0,1,1 loc loc_2011:

while hMDM2N <=dpMDM2N wait dhP53=-1,dhMDM2N=1,dhAKT=-1,dhMDM2C=0,dh=1 when hMDM2N=dpMDM2N do hMDM2N'=0, k'=k+1, l'=l+h, h'=0 goto loc_2111;

– pour la configuration 2,1,0,1 loc loc_2101:

while hP53 >=dnP53 wait dhP53=-1, dhMDM2N=0, dhAKT=0, dhMDM2C =- 1,dh = 1 when hP53=dnP53 do hP53'=0, k'=k+1, l'=l+h, h'=0 goto loc_1101;

– pour la configuration 2,1,1,1 loc loc_2111: while hAKT >= dnAKT wait

dhP53=-1, dhMDM2N=0, dhAKT=-1, dhMDM2C=0 ,dh=1 when hAKT = dnAKT do hAKT'= 0, k'=k+1, l'=l+h, h'= 0 goto loc_2101;

end

– Analysis commands

var

portrait, fstate,nes_cyc_length,pln_cyc_length,fixpoint, r_ini, r_old, r_new, r_acc: region;

r_ini:=loc[auto]=loc_0000 & hP53 >=0 & hAKT >=0 & hMDM2C >=0 & hP53 <=dpP53 & hAKT <=dpAKT & hMDM2C <=dpMDM2C;

– hx >=0 & hx <=dpx0 & hy >=0 & hy <=dpy0;

–r_ini:=loc[auto]=loc_10 & hx >=0 & hx <=dpx1 & hy >=0 & hy <=dpy0;

r_new:=hide k,n in hull (post(r_ini & k=n) & k=n) endhide;

r_old:=r_ini & r_ini;

nes_cyc_length:= h=0 & l=0;

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;

nes_cyc_length:=hide n in hull(post(nes_cyc_length & 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;

nes_cyc_length:=hide n in hull(post(nes_cyc_length & k=n) & k=n) endhide ;

endwhile;

r_acc:=hull(r_acc & r_ini);

r_new:=hull(r_acc & r_new);

```
–print hide non_parameters in r_new endhide;
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 constraints:";
print hide hP53,hMDM2N,hAKT,hMDM2C,h in r_new endhide;
prints "================================";
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;
–Length of an I.K
fixpoint:=r_new;
if not empty(r_new) then –the following algorithm finds the length of the
trajectory in time units
pln_cyc_length:= h=0 & l=0;
portrait:=r_new;
portrait:=portrait — r_acc;
pln_cyc_length:=hide n in hull(post(pln_cyc_length & k=n) &  k=n) end-
hide ;
r_old:=r_ini &  r_ini; –empty region initialization while not r_new=r_old
do
r_old:=r_new;
r_acc:= hide n in hull(post(r_new & k=n) &  k=n) endhide;
```

```
    portrait:=portrait — r_acc;
    r_new:=hull(r_acc — r_new);
    pln_cyc_length:= hide n in hull(post(pln_cyc_length & k=n) &  k=n) end-
hide ;
    endwhile;
    prints "=====================================";
–prints "All regions of the invariace kernel:";
    –print hide h in hull(portrait) endhide;
    prints "=====================================";
prints "Length of a plain cycle is:";
    print hide hP53,hMDM2N,hAKT,hMDM2C,h in hull(pln_cyc_length &
r_ini) endhide;
    –print hide hP53,hMDM2N,hAKT,hMDM2C,h in hull(pln_cyc_length) end-
hide;
    prints "=====================================";
    prints "Length of a nested cycle is:";
    print hide hP53,hMDM2N,hAKT,hMDM2C,h in hull(nes_cyc_length &
r_ini) endhide;
    –print hide hP53,hMDM2N,hAKT,hMDM2C,h in hull(nes_cyc_length) end-
hide;
    prints "=====================================";
endif;
```

Appendix C

HyTech Code-Cycle 31

var

hP53,hMDM2N,hAKT,hMDM2C,h :analog;

k,n,l : discrete;

dpP53, dnP53,dpMDM2N, dnMDM2N, dpAKT, dnAKT, dpMDM2C, dnMDM2C: parameter;

automaton auto

synclabs: ;

initially loc_0010;

– gĉne $n°0$ = P53

– gĉne $n°1$ = MDM2N

– gĉne $n°2$ = AKT

– gĉne $n°3$ = MDM2C

– pour la configuration 0,0,1,0

loc loc_0010:

while hP53 <=dpP53 w a it dhP53 = 1, dhMDM2N = 1, dhAKT =- 1, dhMDM2C = 1, dh=1

when hP53=dpP53 do hP53'=0,k'=k+1,l'=l+h,h'=0 goto loc_1010;

– pour la configuration 0,1,0,0

loc loc_0100:

while hAKT <=dpAKT wait dhP53 = 1, dhMDM2N =- 1, dhAKT = 1, dhMDM2C = 1, dh = 1 when hAKT=dpAKT do hAKT'= 0, k'= k+1, l'=

l+h, h'= 0 goto loc_0110;

– pour la configuration 0,1,0,1

loc loc_0101:

while hMDM2C >=dnMDM2C wait dhP53=0, dhMDM2N=-1, dhAKT=1, dhMDM2C =- 1, dh= 1

when hMDM2N=dnMDM2N do hMDM2N'=0,k'=k+1,l'=l+h,h'=0 goto loc_0100;

– pour la configuration 0,1,1,0

loc loc_0110:

while hMDM2N >= dnMDM2N wait dhP53=1, dhMDM2N=-1, dhAKT=0, dhMDM2C=1, dh=1

when hMDM2N=dnMDM2N do hMDM2N'=0,k'=k+1,l'=l+h,h'=0 goto loc_0010;

– pour la configuration 1,0,1,0

loc loc_1010: while hMDM2C <=dpMDM2C wait dhP53=1,dhMDM2N=1, dhAKT=-1, dhMDM2C=1, dh=1

when hMDM2C=dpMDM2C dohMDM2C'=0,k'=k+1,l'=l+h,h'=0 goto loc_1011;

– pour la configuration 1,0,1,1

loc loc_1011:

while hMDM2N <=dpMDM2N wait dhP53=1,dhMDM2N=1,dhAKT=-1, dhMDM2C=-1, dh=1

when hMDM2N=dpMDM2N do hMDM2N'=0,k'=k+1,l'=l+h,h'=0 goto loc_1111;

– pour la configuration 1,1,0,1

loc loc_1101:

while hP53 >= dnP53 wait dhP53=- 1, dhMDM2N=- 1, dhAKT= 1, dhMDM2C=-1, dh= 1

when hP53=dnP53 dohP53'=0,k'=k+1,l'=l+h,h'=0 goto loc_0101;

– pour la configuration 1,1,1,1

loc loc_1111:

while hAKT >=dnAKT wait dhP53=- 1, dhMDM2N=0, dhAKT=- 1, dhMDM2C =- 1, dh= 1

when hAKT= dnAKT do hAKT'=0, k'= k+1,l' =l+h, h'=0 goto loc_1101;

end

– Analysis commands

var

portrait, fstate, nes_cyc_length, pln_cyc_length,fixpoint, r_ini, r_old, r_new, r_acc: region;

r_ini:=loc[auto]=loc_0010 & hP53 >=0 & hP53<=dpP53; – hx >=0 & hx <=dpx0 & hy >=0 & hy <=dpy0;

–r_ini:=loc[auto]=loc_10 & hx >=0 & hx¡=dpx1 & hy>=0 & hy <=dpy0;

r_new:=hide k,n in hull (post(r_ini & k=n) & k=n) endhide;

r_old:=r_ini & r_ini;

nes_cyc_length:= h=0 & l=0;

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;

nes_cyc_length:=hide n in hull(post(nes_cyc_length & 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;

nes_cyc_length:=hide n in hull(post(nes_cyc_length & k=n) & k=n) endhide ; endwhile;

r_acc:=hull(r_acc & r_ini);

r_new:=hull(r_acc & r_new);

–print hide non_parameters in r_new endhide;

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 constraints:";
print hide hP53,hMDM2N,hAKT,hMDM2C,h in r_new endhide;
prints "=====================================";
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;
–Length of an I.K
fixpoint:=r_new;
if not empty(r_new) then –the following algorithm finds the length of the trajectory in time units pln_cyc_length:= h=0 & l=0;
portrait:=r_new;
portrait:=portrait — r_acc;
pln_cyc_length:=hide n in hull(post(pln_cyc_length & k=n) &  k=n) endhide ;
r_old:=r_ini &  r_ini;
–empty region initialization while not r_new=r_old do
r_old:=r_new;
r_acc:= hide n in hull(post(r_new & k=n) &  k=n) endhide;
portrait:=portrait — r_acc;
r_new:=hull(r_acc — r_new);
pln_cyc_length:= hide n in hull(post(pln_cyc_length & k=n) &  k=n) endhide ;
endwhile;
prints "=====================================";
–prints "All regions of the invariace kernel:";
```

```
–print hide h in hull(portrait) endhide;
prints "==================================";
prints "Length of a plain cycle is:";
print hide hP53,hMDM2N,hAKT,hMDM2C,h in hull(pln_cyc_length & r_ini) endhide;
–print hide hP53,hMDM2N,hAKT,hMDM2C,h in hull(pln_cyc_length) endhide;
prints "==================================";
prints "Length of a nested cycle is:";
print hide hP53,hMDM2N,hAKT,hMDM2C,h in hull(nes_cyc_length & r_ini) endhide;
–print hide hP53,hMDM2N,hAKT,hMDM2C,h in hull(nes_cyc_length) endhide;
prints "==================================";
endif;
```

Appendix D

HyTech Code-Cycle 43

var

hP53,hMDM2N,hAKT,hMDM2C,h :analog;

k,n,l : discrete;

dpP53, dnP53, dpMDM2N, dnMDM2N, dpAKT, dnAKT, dpMDM2C, dnMDM2C: parameter;

automaton auto

synclabs: ;

initially loc_0010;

– g$\hat{c}ne$ $n°0$ = P53

– g$\hat{c}ne$ $n°1$ = MDM2N

– g$\hat{c}$ne $n°2$ = AKT

– g$\hat{c}$ne $n°3$ = MDM2C

– pour la configuration 0,0,1,0

loc loc_0010:

while hMDM2C <=dpMDM2C wait dhP53=1,dhMDM2N=1,dhAKT=-1,dhMDM2C=1, dh=1

when hMDM2C=dpMDM2C do hMDM2C'=0, k'=k+1,l'=l+h,h'=0goto loc_0011;

– pour la configuration 0,0,1,1

loc loc_0011:

while hP53<= dpP53 wait dhP53 = 1, dhMDM2N=1, dhAKT=-1,

dhMDM2C = 0, dh = 1

when hP53=dpP53 do hP53'=0,k'=k+1,l'=l+h,h'=0 goto loc_1011;

– pour la configuration 0,1,0,0

loc loc_0100:

while hAKT <= dpAKT wait dhP53 = 1, dhMDM2N =- 1, dhAKT = 1, dhMDM2C = 1, dh= 1

when hAKT=dpAKT do hAKT'=0,k'=k+1,l'=l+h,h'=0 goto loc_0110;

– pour la configuration 0,1,0,1

loc loc_0101:

while hMDM2C >=dnMDM2C wait dhP53=0,dhMDM2N=-1,dhAKT=1, dhMDM2C=-1, dh=1

when hMDM2C=dnMDM2C do hMDM2C'=0,k'=k+1,l'=l+h,h'=0 goto loc_0100;

– pour la configuration 0,1,1,0

loc loc_0110:

while hMDM2N >=dnMDM2N wait dhP53=1,dhMDM2N=-1,dhAKT=0, dhMDM2C=1, dh=1

when hMDM2N=dnMDM2N do hMDM2N'=0, k'=k+1,l'= l+h, h'=0 goto loc_0010;

– pour la configuration 1,0,1,1

loc loc_1011:

while hP53 <= dpP53 wait dhP53 = 1, dhMDM2N = 1, dhAKT =-1, dhMDM2C =- 1, dh=1

when hP53=dpP53 do hP53'=0,k'=k+1,l'=l+h,h'=0 goto loc_2011;

– pour la configuration 1,1,0,1

loc loc_1101:

while hP53 >= dnP53 wait dhP53 =- 1, dhMDM2N =- 1, dhAKT = 1, dhMDM2C =- 1, dh = 1

when hP53=dnP53 do hP53'=0,k'=k+1,l'=l+h,h'=0 goto loc_0101;

– pour la configuration 1,1,1,1

loc loc_1111:

while hAKT >= dnAKT wait dhP53 =-1, dhMDM2N = 0, dhAKT =-1, dhMDM2C =- 1, dh = 1

when hAKT=dnAKT do hAKT'=0,k'=k+1,l'=l+h,h'=0 goto loc_1101;

– pour la configuration 2,0,1,1

loc loc_2011:

while hMDM2N <=dpMDM2N wait dhP53=-1,dhMDM2N=1,dhAKT=-1,dhMDM2C=0, dh=1

when hMDM2N=dpMDM2N do hMDM2N'=0,k'=k+1,l'=l+h,h'=0 goto loc_2111;

– pour la configuration 2,1,1,1

loc loc_2111:

while hP53 >= dnP53 wait dhP53 =- 1, dhMDM2N=0, dhAKT=-1, dhMDM2C = 0, dh=1

when hP53=dnP53 do hP53'=0,k'=k+1,l'=l+h,h'=0goto loc_1111;

end

– Analysis commands var portrait,fstate,nes_cyc_length,pln_cyc_length,fixpoint,r_ini,r_old, r_new,r_acc: region;

r_ini:=loc[auto]=loc_0010 & hMDM2C >=0 & hMDM2C¡=dpMDM2C; – hx¿=0 & hx<=dpx0 & hy >=0 & hy <=dpy0;

–r_ini:=loc[auto]=loc_10 & hx >=0 & hx <=dpx1 & hy >=0 & hy <=dpy0;

r_new:=hide k,n in hull (post(r_ini & k=n) & k=n) endhide; r_old:=r_ini & r _ini;

nes _cyc _ length:= h=0 & l=0;

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;

nes_cyc_length:=hide n in hull(post(nes_cyc_length & 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; nes_cyc_length:=hide n in

hull(post(nes_cyc_length & k=n) & k=n) endhide ;

endwhile;

r_acc:=hull(r_acc & r_ini);

r_new:=hull(r_acc & r_new);

–print hide non_parameters in r_new endhide;

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 constraints:";

print hide hP53,hMDM2N,hAKT,hMDM2C,h in r_new endhide;

prints "==========";

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;

–Length of an I.K

fixpoint:=r_new;

if not empty(r_new) then –the following algorithm finds the length of the trajectory in time units

pln_cyc_length:= h=0 & l=0;

portrait:=r_new;

portrait:=portrait — r_acc;

pln_cyc_length:=hide n in hull(post(pln_cyc_length & k=n) & k=n) endhide ;

r_old:=r_ini & r_ini; –empty region initialization while not r_new=r_old do r_old:=r_new;

r_acc:= hide n in hull(post(r_new & k=n) & k=n) endhide;

portrait:=portrait — r_acc;

r_new:=hull(r_acc — r_new);

pln_cyc_length:= hide n in hull(post(pln_cyc_length & k=n) & k=n) endhide ;

endwhile;

prints "==========";

–prints "All regions of the invariace kernel:";

–print hide h in hull(portrait) endhide;

prints "==========";

prints "Length of a plain cycle is:";

print hide hP53,hMDM2N,hAKT,hMDM2C,h in hull(pln_cyc_length & r_ini) endhide;

–print hide hP53,hMDM2N,hAKT,hMDM2C,h in hull(pln_cyc_length) endhide;

prints "==========";

prints "Length of a nested cycle is:";

print hide hP53,hMDM2N,hAKT,hMDM2C,h in hull(nes_cyc_length & r_ini) endhide;

–print hide hP53,hMDM2N,hAKT,hMDM2C,h in hull(nes_cyc_length) endhide;

prints "==========";

endif;