Formal Modeling and Analysis of Cell Cycle Regulator E2F1 and Micro RNA-223 in Acute Myeloid Leukemia

By

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Declaration

I thus proclaim that the work exhibited in the following thesis is my own effort, with the exception of where generally acknowledged, and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Sara Rafiq

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Ameen

Sara Rafiq

Abstract

CCAAT enhancer binding protein alpha (C/EBPα), is a fundamental transcription factor for granulopoiesis (formation of granulocytes i.e. white blood cells) but its expression is downregulated in leukemia. Downregulation of E2F1, the expert controller of myeloid cell proliferation by C/EBP α is significant for granulopoiesis as it is involved in activation of microRNA-223 (miR-223). Latter studies show that miR-223, a transcriptional focus of C/EBP α, acts as a discriminating player during granulopoiesis and is downregulated in distinctive subtypes of Acute Myeloid Leukemia. Discrete modeling formalism of René Thomas is a well-known methodology utilized for demonstration and examination of Biological Regulatory Networks (BRNs). Logical parameters for the BRN were induced utilizing René Thomas formalism, executed in SMBioNet. Discrete modeling of the BRN is further carried out to foresee behaviors which either prompt ordinary granulocytic differentiation or differentiation blockage using GENOTECH tool. The results obtained from GENOTECH suggest that out of 16 models, the models obtained from multivalued boolean logic (e.g. BRN named as 12121) provided better results rather than models obtained from boolean formalism. Further on, the results generated from these models suggest that downregulation of E2F1 because of increased threshold levels of C/EBPα and miR-223 can lead towards normal granulopoiesis instead of uncontrolled proliferation (the myeloid cells). The delay constraints are computed by using hybrid model (Bio-Linear Hybrid Automaton) which characterize the homeostasis of the BRN. These findings suggest that E2F1

can be potential therapeutic target for AML.

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Acronyms

Bio-LHA Parametric Biological Linear Hybrid Autotmata

BRN Biological Regulatory Network

CTL Computational tree logic

HyTech The Hybrid Technology tool

PARA Parametrization

AML Acute Myeloid Leukemia

C/EBPα Ccaat-Enhancer-Binding Protein Alpha

miRNAs Micro RNAs

TFs Transcription Factors

miR-223 Micro RNA-223

SMBioNet Selection of Models of Biological Networks

Chapter 1

Introduction

1.1 Cancer

Cancer is a class of ailments described by immature cell development. Each type of cancer is characterized by the sort of cell that is at first influenced. Cancer damages the body when immature cells isolate destructively to forms swellings and tumors (aside from leukemia where disease restricts typical to blood by irregular cell division in the circulatory system) [1, 2]. Tumors can develop and meddle different biological systems, and they can secrete hormones that change body functions. Tumors that stay at one spot and exhibit constrained development are said to be benevolent [3].

Most risky, or harmful, tumors occur when two conditions happen:

- a cancerous cell gets by all through the body utilizing the blood or lymphatic system, destroying favorable tissues in a process known as intrusion [4]

- cancerous cell figures out how to duplicate and develop, making blood vessels to nourish itself in a mechanism called angiogenesis [5]

At the point when a tumor effectively spreads to different body parts and develops, attacking and obliterating other normal tissues, the phenomenon is clas-

sified as being metastasized [6, 7] . This procedure itself is known as metastasis, and the result is a drastic state that is exceptionally hard to treat [8].

1.2 Diagnosis of Cancer

As earlier the malignancy is spotted and treated, the greater are the chances of its being cured. A few types of tumor, for example, skin, breast, testicles, rectum, prostate and mouth may be caught by normal examination towards oneself or other screening measures before the tumor gets severe. Most cases of malignancy are caught and diagnosed when altered indications are observed or after a tumor can be sensed. In few cases, malignancy is diagnosed by chance as an after-effect of treatment of other therapeutic conditions [9].

Cancer diagnosis begins with a thorough physical examination and a wide-ranging restorative history. Blood and stool lab reports can catch differences from the normal that may demonstrate cancer. At the moment of suspection of tumor, imaging tests such as CT scan, MRI, fiber-optic endoscopy and ultrasound examinations aid specialists to concentrate over the malignancy's area and size. To confirm the conclusion of most malignancies, a biopsy is to carried out in which a specimen of tissue is expelled from the suspected tumor and is studied under a microscope to report for malignant cells [1, 10].

1.3 Advancement of Cancer

Certain interactions amongst different types of cells and the extracellular matrix that holds them together cause them to detach at the tumor site, they get expelled and reattach themselves at another site. It is considered that this revelation is critical in light of the fact that growth mortality is predominantly because of metastatic tumors that develop from cells that move from their unique site to an

alternate place in the body. Just 10% of cancerous cases are created by the primary tumors [11]. Figuring out how to prevent malignant cells from adhering to new destinations could meddle up with metastatic ailment, and end the development of auxiliary tumors [8].

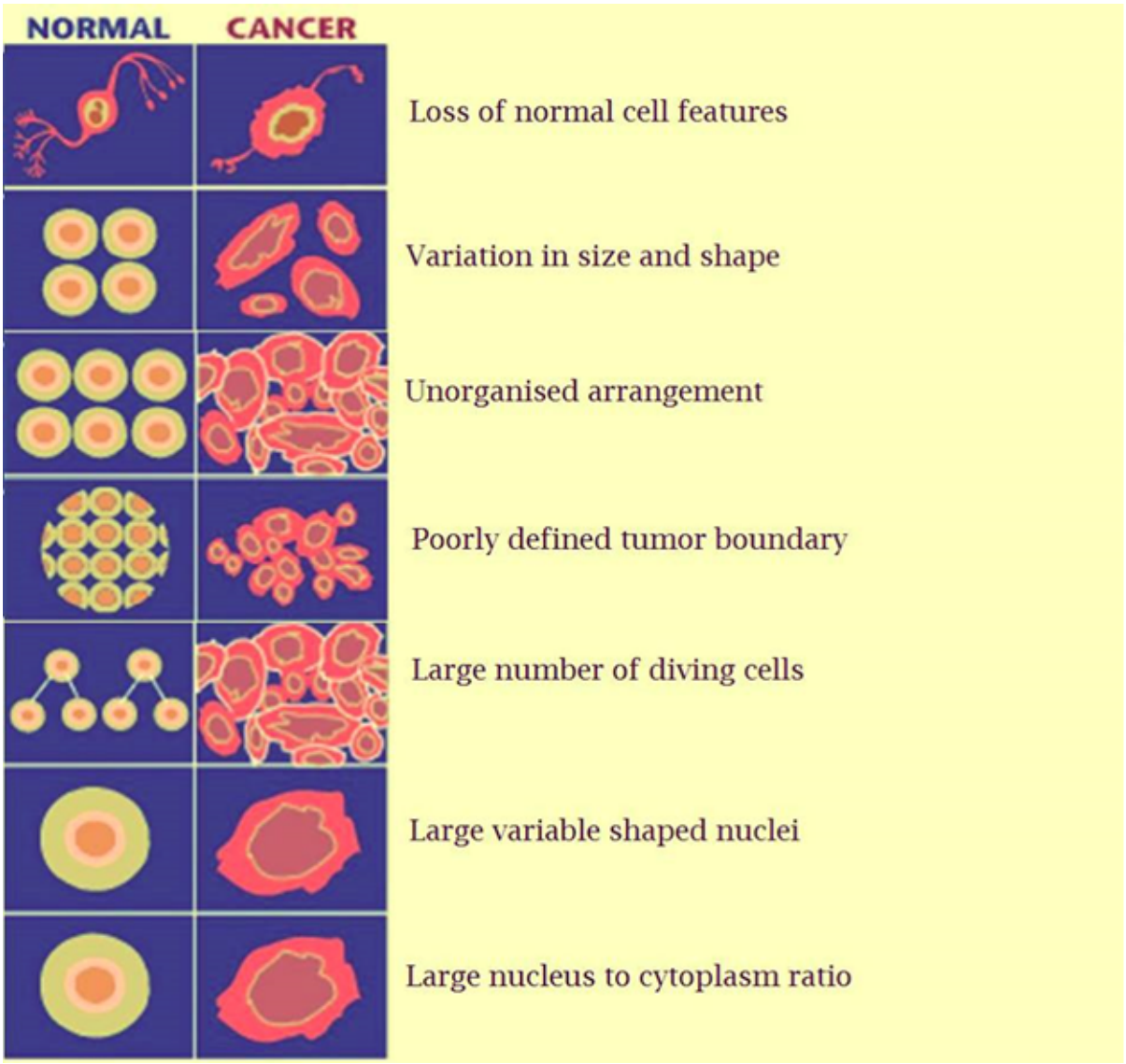

Figure 1.1: Normal and Cancerous Cells. Most common differences between normal and cancerous cells [1].

1.4 Causes of Cancer

Cancer is eventually the after-effect of cells which wildly develop and do not cease to duplicate. Ordinary body cells follow an organized way of development, division, and death. Apoptosis is defined as a programmed cell death, and when this procedure is interrupted, malignant tumors are formed. Contrary to normal cells, cancer cell do not encounter apoptosis and rather keep on growing and multiply rapidly. This prompts a mass of irregular cells that keeps on multiplying [8].

1.5 Types of Cancer

There are more than 200 sorts of cancer. There are sixty separate organs in the body where a malignancy can develop. Out of them blood cancer is one of the most important type:

1.5.1 Blood Cancer

Blood cancer affects the production and function of different blood cells. Typically blood cells are produced in bone marrow. Stem cells present in bone marrow mature and grow into three types of blood cells i.e. red blood cells, white blood cells and platelets. Generally in cancers the production of normal blood cells is interrupted by uncontrolled growth of an immature or abnormal blood cell. This abnormal mass of cells prevents normal body functions. Cancerous cell can assault any natural process of the human physiology. As a feature of blood cancer, the quickly increasing dangerous cells are discovered assaulting the distinctive parts of the circulatory system. Other than blood and the lymphatic system; the bone marrow can likewise be the center of attack.

1.5.2 Types of Blood Cancer

Fundamentally, there are three forms of blood cancer. Each of the cancer likewise include a few varieties, however as a rule this growth is sorted into the accompanying sorts:

- **Leukemia**- With spurt in the abundance of cancerous cells influencing either the marrow or the blood, the capability of the circulatory framework to create blood is extremely hindered.

- **Lymphoma**- The tumor cells influencing the lymphocytes are known as the lymphoma. Lymphocytes are one of the mixed bags of white blood corpuscles.

- **Myeloma**- In general as a part of Myeloma, the plasma (an alternate mixed bag of WBC) is influenced by the cancerous cells.

Amongst them the most common type of blood cancer is Leukemia. Leukemia belongs to the type of cancer that affects white blood cells. It is regarded as overproduction of immature WBCs called blasts involving an abnormal production of granulocytes, myelocytes, and myeloblasts in the circulating blood. Generally, Leukemia term is used for a number of diseases affecting blood, bone marrow (specially referred to myeloid tissues), spleen and typically lymphoid system also called hematological neoplasms.

So the basic features characterizing leukemia are:

- Ability to proliferate continuously

- Due to mutations affecting growth factors

- Transcription errors

- Arrested development of normal cells

1.5.3 Symptoms

Leukemia is marked by an acute loss of healthy red blood corpuscles and incorporates the indications of anemia, frequent faintness and compelling exhaustion. Hence one influenced by it is liable to sweat and go under shortness of breath in course of performing everyday exercises of the normal kind. Powerlessness to disease and swelling of the lymph nodes are a portion of symptoms of Leukemia. Blood tests are expected to present higher counts of white blood cells. Leukemia might be acute or chronic. An individual distressed with the former type may not show any of these side effects. Then again, in leukemia of the intense sort, the indications are prone to show with rapid acuteness.

There are four basic types of leukemias:

- Acute myeloid leukemia (AML)
- Chronic myeloid leukemia (CML)
- Acute lymphocytic leukemia (ALL)
- Chronic lymphocytic leukemia (CLL)

An alternate kind of leukemia, hairy cell leukemia, is an uncommon subtype of chronic lymphocytic leukemia (CLL). It is created by an expanded number of lymphocytes and advances gradually. It is called "hairy " on account of its distinct appearance of cells during *invitro* analysis. The essential contrasts between the four basic sorts of leukemia need to do with their rates of movement and where the disease starts. Chronic leukemic cells do not develop fully, so they are not as equipped for shielding against contaminations as typical lymphocytes. Acute leukemic cells start to duplicate before any immune response gets developed [12]. Our main focus of study is on molecular pathogenesis of acute myeloid leukemia (AML) which will be discussed in further sections.

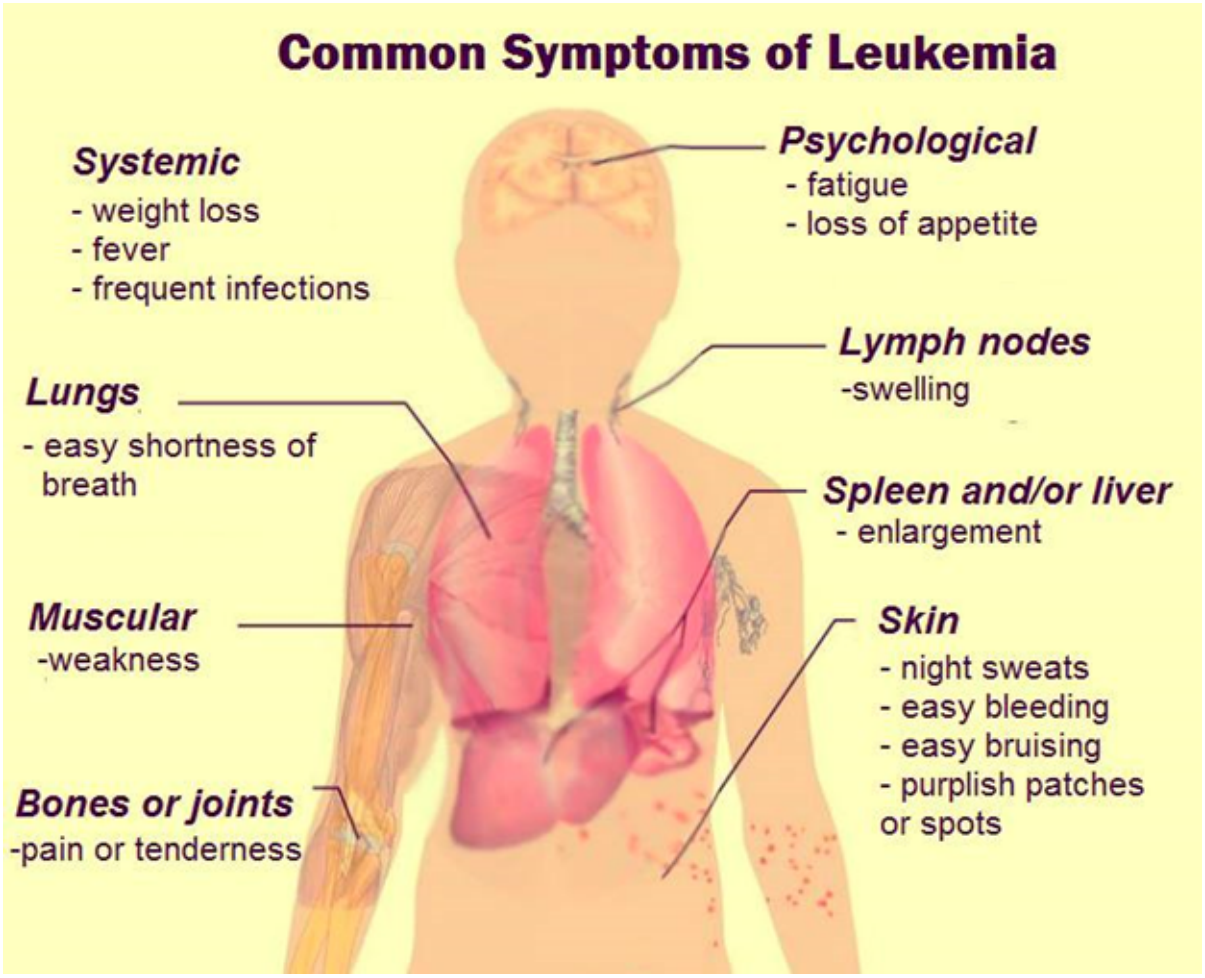

Figure 1.2: Symptoms of Acute Myeloid Leukemia. The most common symptoms of Leukemis are weakness, shortness of breath, pain, fatigue, loss of appetite, swelling and night sweats [1].

1.5.4 Problem Statement

In spite of the fact that AML is generally an uncommon disease, representing roughly 1.2% of malignancy deaths in the United States [13], its frequency is observed to get increased as the population increases. Therefore, by modeling different transcription factors related to it we can predict notable therapeutic techniques to fight against this disease and modeling of its molecular pathway provides us a better understanding of its causes and the factors that should be considered to avoid such drastic disease.

1.6 Theme of Study

The essential theme of study is the application of formal techniques, for example logical formalism and model checking on the transcription factors involved in AML and their impact on malignancy at different threshold levels. Overall, the study focuses on the computational methodologies of systems biology and network biology in the modeling and analysis of these procedures so as to guide the behavioral inclinations of the included proteins.

Chapter 2

Literature Review

2.1 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) symbolizes a grouping of clonal hematopoietic stem cell in which loss of functionality related to overproliferation and differentiation in the undifferentiated cells, bring about collection of malfunctioned cells termed myeloblasts. While the particular reason for this natural irregularity in any individual patient is generally obscure, the thriving understanding of the hereditary underpinnings of leukemia is starting to prompt a wide number of treatments, huge numbers of which are in clinical improvement [14].

2.2 Epidemiology

The yearly occurrence of AML in infants up to 15 years old is evaluated to be between 507 cases for every million infants at danger, representing 15.20 % of all childhood leukemias [15]. The top frequency of infant AML happens in the first year of existence with 30% of childhood AML patients being patinger than 2 years at examination. The rate remains relatively steady from 3 years of age all through early age and adulthood. There is a slight female transcendence in babies

following a higher frequency in males, however no solid sex particular contrast in occurrence is observed.

2.3 Molecular Basis of AML

The essentials of particular cytogenetic injuries as capable determinants of the remedial reaction proposes that different transformational mechanisms connected with these injuries are likely to specifically impact the affectability of the leukemic blast cells. It is important that we can comprehend why certain cytogenetic injuries are connected with a favorable result, we may have the capacity to apply this learning to enhance the therapeutic methodology among patients with AML. The recent research on hereditary focuses basic AML-related cytogenetic anomalies and the illustration of their components of activity have started to give discriminating experiences into this issue.

The most well-known targets of AML-related chromosomal translocations are genes that encode DNA transcriptional complexes. Transformation in each of these cases seems to come about because of the generation of translocated proteins that meddle in an overwhelming way with the function of the wild protein. Investigation of three particular genetic lesions has given basic experiences into the pathogenesis of AML and has effectively served to recognize subgroups for therapies [16].

Table2.1 demonstrates the most continuous chromosomal translocations in myeloid malignancies. Commonly translocations are said to promote the combination of two genes encoding factors associated with transcription. These factors show conservation during evolution, and are vital in embryonic development and additionally in ordinary hematopoiesis. TFs included in AML incorporate core binding factor (CBF), retinoic acid receptor alpha (RARa), homeobox (HOX) family, and mixed lineage leukemia (MLL).

Table 2.1: Chromosomal abnormalities related to Aute Myeloid Leukemia

Chromosome Abnormality	Disease	Fusion Gene
t(6;11)	AML-M5	*AML-M5*
t(10;11)		*MLL-AF6AF6q21*
t(11;17)		*MLL-AF10;CALM -AF10*
t(11;19)		*MLL-AF17/AF17q25*
t(4;11)		*MLL-AF4*
t(8;21)(q22;q22)	AML-M2	*AML1-ETO*
t(15;17)(q21-11-22)	AML-M3	*PML-RARalpha*
t(11;17)(q23;q21)	AML-M3	*PIZF–RARalpha*
t(9;11)(p22-q23)	AML-M4	*MLL-AF9*

2.4 Transription Factors involed in AML

2.4.1 CCAAT/enhancer binding proteins (C/EBPs)

Recently, a connection between the transcription factors of gene expression and proliferation cycle has been identified. A fundamental type of these transcription factors is the CCAAT/enhancer binding protein (C/EBP) group of basic leucine zipper domain proteins. These transcription factors control the differentiation process of stem cells, and have key parts in controlling cell multiplication through connection with cell cycle proteins. Their significance during the time of differentiation and proliferation has made them significant controllers during tumorigenesis [17].

2.4.2 MicroRNA 223

MicroRNAs (miRNAs) belong to the novel class of non-coding RNAs. These are expressed widely in animals and plants in order to regulate post transcription gene regulation either by cleavage or translational repression of their specific target mRNAs. miRNAs range about 21-25 nucleotides in length. They play a significant role on control of gene expression in different biological processes at specific stages. They are an important cell regulator as affecting almost every

function of cell, for example, antiviral defense, cell differentiation, development, survival and apoptosis [18].

MicroRNA-223 (miR-223) can upregulate ordinary granulopoiesis. miR-223 functionality is managed by two unique C/EBPα positions. It is reported that miR-223 is suppressed to a great extent in individuals suffering from AML. By sequencing, it is found that miR-223 concealment in AML is not brought on by DNA changes, nor it is intervened by promoter hypermethylation. Results propose that miR-223 concealment in AML is brought on by its interaction with another transcription factor named E2F1 [19].

2.4.3 E2F1

E2F1 is a protein (transcription factor) that in homosapiens is encoded by the E2F1 gene. This protein is a part of the E2F group of TFs. The E2F family plays an important part in the control of cell cycle and in activity of tumor silencing proteins and additionally focuses on different proteins related to DNA viruses causing tumors.

Later studies demonstrate the capability of E2F1 to control miRNAs also how this could be important in the pathogenesis of certain malignancies. E2F1 has been indicated to meddle with myeloid cell differentiation and also seen to promote myeloid progenitors proliferation.

2.4.4 Role of Transcription Factors during AML

CCAAT enhancer binding protein α (C/EBP α) works as a key arbitrator of granulopoiesis. It is observed that in mice C/EBPα silencing demonstrates a particular deadlock in the differentiation of granulocytes. In AML, C/EBPα is deregulated by different procedures including its transformations. Restraint of E2F function-

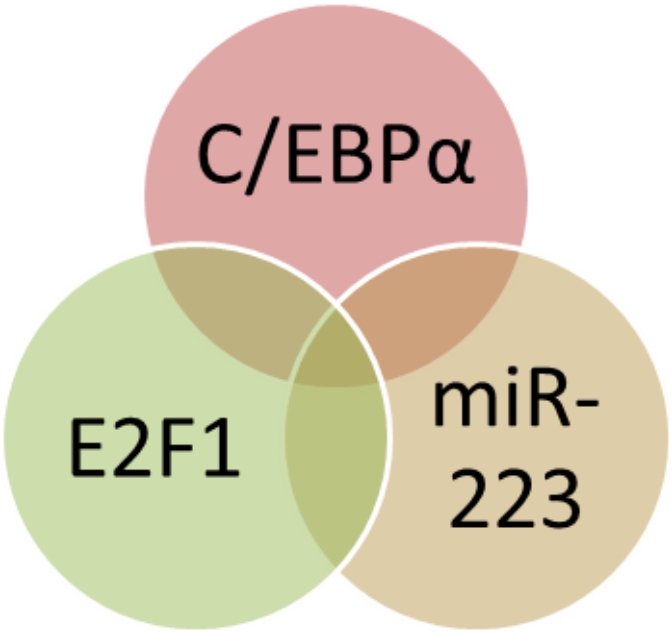

Figure 2.1: Transcription Factors involved in AML. Entities involved in Acute Myeloid Leukemia are C/EBPα, E2F1 and miR-223.

ality by C/EBPα is a key point for the antimitotic functionality of C/EBPα in granulopoiesis. Targeted interruption in C/EBPα required for E2F binding brings about block of granulopoiesis in mice. Study reveals that, mice convey a germline transformation that deregulates C/EBPα linked E2F suppression, develop AML. Interestingly, E2F1 has the capacity to repress granulopoiesis and force myeloid cell-cycle movement. On the other hand, there has been no complete mechanism showed for C/EBPα interceded E2F1 suppression in granulopoiesis. miRNAs is a novel family of gene regulators and are observed to play key roles in biologic mechanisms, for example, cell differentiation, proliferation and apoptosis, all of which are often influenced in AML. Developing number of studies exhibit that the deregulation of miRNAs is connected with the advancement of numerous malignancies incorporating leukemia. In granulopoiesis, microRNA-223 (miR-223) is a standout element amongst the most discriminating miRNAs. miR-223 has been demonstrated to be regulated by myeloid TFs, for example, C/EBPs and PU.1. The vital part of miR-223 in granulopoiesis was demonstrated by a late finding

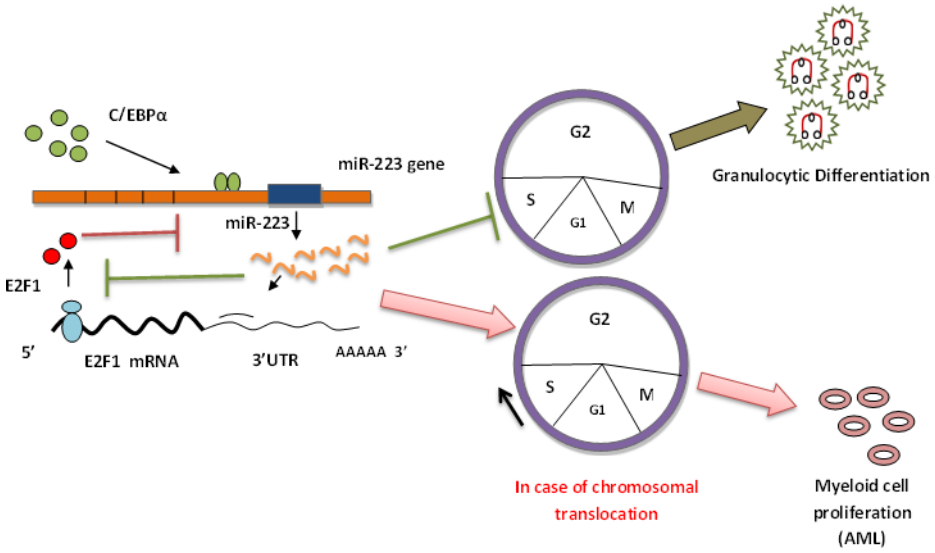

Figure 2.2: Model for the role of C/EBPα, miR-223 and E2F1. Top panel shows that C/EBPα transactivates miR-223 promoter which in return represses E2F1 activity and induce granulocytic differentiation. Bottom panel shows deactivation of C/EBPα which results in accumulation of E2F1 and favors myeloid cell proliferation as over expression inhibits miR-223 transcription [20].

that mice having dearth of miR-223 show abnormal granulopoiesis. Interestingly, miR-223 is inactivated by the oncoprotein AML1/ETO in AML.

Deep analysis provides new bits of knowledge to the C/EBPα mediated hindrance of myeloid cell-cycle pathway, which is a key step disturbed in diverse subtypes of AML. In this study, we demonstrated that C/EBPα positively influences miR-223, and miR-223 interrupts myeloid cell cycle by focusing on E2F1. We cannot exclude the plausibility of miR-223 focusing on other cell-cycle controllers during granulopoiesis. The overexpressed E2F1 could bind to the miR-223 promoter and thus prompt a further decay in miR-223 product which is represented by a negative feedback loop thus promoting progression of myeloid cell-cycle. Overexpression of E2F1 has been indicated to be an oncogenic occasion that inclines cells to transform [21, 22].

2.5 General Treatments for Acute Myeloid Leukemia

The fundamental treatment for AML is chemotherapy. Different medicines for AML incorporate growth factors, radiotherapy, and bone marrow or stem cell transplants. The treatment of AML changes relying upon its kind, patient's fitness, and patient's age and level of health.

2.5.1 Induction

The point of this treatment is to kill the leukemia cells. It is called remission induction. After remission there is no indication of leukemia in patient's blood or bone marrow. You may need to stay under doctor's administration for a month. Before pati begin chemotherapy patient may need to have blood transfusions and platelet transfusions. After that patient is treated with chemotherapy [23].

2.5.2 Consolidation

Once there is no indication of the leukemia, patient have consolidation treatment to stop it returning once more. Consolidation treatment may mean

- More chemotherapy
- A transplant
- A transplant with patient's blood undifferentiated stem cells (this is infrequently utilized for AML)

2.6 Graphical Models

A graphical model using parametric biological linear hybrid automata (Bio-LHA) was developed which represented the molecular connections as a biological regulatory network (BRN) improved with parametric time delays. This methodology

was a mixture of discrete and continuous domain in which the diverse oscillatory trajectories of the AML were represented while evaluating the time delays eventually leading to those states.

2.7 Aims and Objectives

The above mentioned figure 2.2 does not represent that at which threshold values these three transcription factors target each other during AML or normal conditions. Therefore, we proposed a graphical modeling approach with the aim of showing the links between these three transcription factors. The specific objectives of the proposed methodology are

- Construction of Biological Regulatory Network
- Modeling of observations using CTL formulas and then analyzing their dynamics with discrete and hybrid formalisms
- Inferring qualitative model based on observations
- Analysis of critical paths and expression dynamics of entities of BRN
- Refinement of qualitative model by incorporating time delays
- Construction of linear hybrid model of BRN
- Analysis of hybrid model for invariance kernel

Chapter 3

Materials and Methods

3.1 Dynamic model

A network is a potent tool in system biology to explore the essential biological problem, for example complex disease occurrence and development pathway and the current cell reprogramming. Networks can be exploited to determine novel genes that play role in specific biological processes since cellular functions rely on physical, genetic and other types of interactions. Network dynamics is the study of network's behaviors that change over time [24]. It is utilized to study the mechanism of complex biological pathways. For instance, living cells respond to apoptosis signals is crucial for appropriate development and maintenance of hemeostasis of body. *Chang.Gu, et al.* explored TNF provoked apoptosis, and developed a mathematical model devoid of the bi-stability requirement. Their simulation discovered a pulse increasing of caspase-3 activation following signaling stimulation to activate the irreversible death program, which is in accordance with the experimental observations. The dynamic model (real time model) is used to model and express the behavior of the system over time. The description involves equations explaining the time dependence of all state variables of the system. To demonstrate a biological network as a dynamical system, identification

of the variables (signaling molecules, protein species) involved, how they interact (network connections), and state variables' values and their interactions changing over time is required [25]. Current progress in high-throughput techniques have generated large amount of biological data reflecting different interactions at various stages. A cell reacts to stimuli in its environment including interactions and regulation at the gene, protein or metabolism level via synchronized biological processes. For instance, the proteins involved in transcription regulation either induce or inhibit the transcription of genes, resulting in an increase or decrease of expanse of mRNA expression. In return, this alters the levels of proteins in a cell leading to a different phase, or possibly a new response of cell. Hence a complex network of interactions is formed by the genes and their products, in which each component can positively or negatively affect the behavior of other component in the system. Proteins are part of intricate interactions as they form large complexes that play an important role in post-translational modification of other proteins. A biological system comprises of huge number of elements (i.e., genes, proteins and metabolites) and their intricate interactions could be represented in a unified form through network modeling. In network modeling, the nodes (vertices) represent the components of the system, while edges (links) represent the interactions between the nodes. In a network, edges are placed according to the direction of information flow or mass transfer. The positive sign in the interaction represents activation whereas, the negative sign signifies inhibition. These processes at diverse stages can be presented as networks of signal transduction and, biological regulatory networks (BRNs) and, protein–protein interaction networks and, and metabolic networks and, respectively. Principally, BRN nodes comprises of genes, mRNAs, and proteins, whereas the edges symbolize transcription, translation, and protein–protein interactions. Biological system consists of complicated network of interactions and interpreting the dynamics of these networks is the initiative towards cell behavior. The representation of network offers a detailed structural

examination and dynamic modeling of the biological system under study. Structural analysis utilizes graph-theoretical measures, for instance shortest paths and centrality measures, to mine information about the heterogeneity and network organization. In dynamic models, nodes are the molecular species in a network that explain variation in population intensities of these species with the passage of time. Dynamic modeling methods are categorized into two main types, i.e. continuous or discrete, depending on the depiction of nodes' states. Continuous models are illustrated as differential equations sets, hence is the supreme approach to understand the dynamics of biological systems. Though, using such models is vulnerable to the inadequacy of the kinetic parameters [26]. Alternatively qualitative description can be achieved by discrete models, for example finite state logical models parameters [27]. The space between discrete and continuous models can be covered by piece-wise linear differential equation (hybrid) models which connects them by illustrating each node with two variables, a continuous concentration and a discrete activity. Regulatory relationships can be logically described with linear concentration decay that can be modeled through the illustrated approaches. Model selection relies totally on the amount of measurable aspects of the experimental facts existing: continuous models could be used when adequate kinetic data exists, discrete models are appropriate for systems which are poorly characterized with kinetic particulars, and hybrid models are implemented when limited data about kinetic parameters is obtainable.

In short a directed graph is defined as follows:

Definition 1 (Directed Graph). *"A directed graph G(N,E) is a tuple, where*:

- *set of all nodes is N and*
- *set of ordered pair of edges or arcs is denoted by* $E \subseteq N \times N$

(e_i, e_j) *is an edge which is considered to be directed from* e_i *to* e_j*, where* e_i *is*

called the head and e_j is called the tail. In G(N,E), $G^-(e)$ and $G^+(e)$ denote sets of predecessors and successors, respectively, of a node $e \in N$. N respectively." [28]

3.2 Biological/Gene regulatory Network

A biological regulatory network (BRN) is a set of relations among biological entities (e.g., proteins in a biological signaling network). The interactions can be either positive (activation) or negative (inhibition). BRNs control the rate at which transcription of genes into mRNA takes place and interaction between genes and proteins in a cell. BRN is used to investigate dynamical behavior of proteins in a biological system.

A BRN is defined as :

Definition 2 (BRN). *"A biological regulatory network (BRN) is a labeled directed graph G(N,E), where the set of nodes N represents biological entities and interactions between entities are represented by the set of edges $E \subseteq N \times N$ in which each edge $e_i \rightarrow e_j$ is labelled by a couple $(j_{e_ie_j}, \eta_{e_ie_j})$, $j_{e_ie_j}$ being a positive integer representing qualitative threshold and $\eta_{e_ie_j}$ being either (+) or (-) sign representing activation or inactivation, respectively. Moreover, we define*:

- *The outdegree of e_i is the total number of targets of e_i, it is denoted by p_{e_i}*
- *Set $Z_{e_i} = \{0, 1, ..., r_{ei}\}$ of an entity e_i represents its qualitative levels (concentration levels) where $r_{e_i} = max\{j_{e_i,e_k} \mid e_k \in G^+(e_i)\}$."* [28]

However, BRN itself is a static diagram, and in spite of the fact that it demonstrates the element prerequisites for the interactions, it cannot determine to the trajectories of BRN behaviors. Such dynamics are rather represented by the state graph produced against a specific set of parameters deduced by the behaviors of entities as a function of resources available for that entity in a given state. The states, resources and the state graphs are formally defined as:

Definition 3 (Qualitative States). *"A qualitative state $s \in S$ of a BRN G(N,E) is a tuple which denotes a configuration of the qualitative expression levels of all entities. The set of states is* :

$$S = \prod_{e_i \in N} Z_{e_i}$$

Vector $(s_{x_{e_i}})_{\forall e_i \in N}$ represents the qualitative states, where level of expression (concentration) of a product e_i is denoted by x_{e_i} [29] "

Definition 4 (Resources). *"At level x_{e_i} , the set of resources $Q_{x_{e_i}}$ of an entity $e_i \in N$ is defined as $\{Q_{x_{e_i}} = e_j \in G^-(e_i) | (x_{e_j}) \geq j_{e_j e_i}$ and $\eta_{e_j e_i} = (+)$ or $(x_{e_j} < j_{e_j e_i}$ and $\eta_{e_j e_i} = (-)\}$*

In the above definition, it is to be noted that the inhibitor is considered as an activator in its absence.

One associates with each qualitative state, the set of resources of each entity which corresponds to the set of predecessors which effectively control the entity (either a present activator or an absent inhibitor).

Definition 5 (Logical Parameters). *"Logical parameters influence the dynamic behavior of a BRN. The set of logical parameters are defined as $K(G) = \{K_{e_i}(Q_{x_{e_i}}) \in Z_{e_i} \forall e_i \in N\}$ [29]."*

Definition 6 (Evolution Operator). *"The evolution operator $\Rsh$ is defined as,*

$$x_{e_i} \Rsh K_{e_i}(Q_{x_{e_i}}) = \begin{cases} x_{e_i} + 1 & \text{if } x_{e_i} < K_{e_i}(Q_{x_{e_i}}); \\ x_{e_i} - 1 & \text{if } x_{e_i} > K_{e_i}(Q_{x_{e_i}}); \\ x_{e_i} & \text{if } x_{e_i} = K_{e_i}(Q_{x_{e_i}}). \end{cases}$$

where x_{e_i} and $K_{e_i}(Q_{x_{e_i}}) \in \{0\} \bigcup \mathbb{N}$. [29]"

Definition 7 (State Graph). "Let G(N,E) be a BRN, let $s = (s_{x_{e_i}})$ be a state and consider the family of logical parameters K(G). The state graph of

the model is the directed graph noted G = (S, T) where S is the set of states and $T \subseteq S \times S$ is the relation between states called transition relation such that $s \to s' \in T$ if and only if:

1. $\ni$ unique $e_i \in N$ such that $s_{x_{e_i}} \neq s'_{x_{e_i}}$ and $s'_{x_{e_i}} = s_{x_{e_i}} \Rsh K_{e_i}(Q_{x_{e_i}})$, and

2. $\forall e_j \in N \backslash \{e_i\} s'_{x_{e_j}} = s_{x_{e_j}}$ [29] ".

3.2.1 Computaional Tree Logic

A CTL formula expresses a dynamical property that is applicable on all the asynchronous state transition graphs resulting from monotonous parameterizations of the network.

The state graph is considered when every state of the graph satisfies the formula; the satisfactory relation between a state and a formula being defined as follows. Firstly, if the formula is merely a propositional formula (that does not comprise temporal operators for example EX or AG), then the satisfactory relation is normal. Secondly, a state O fulfills a CTL formula of the forms mentioned below

EX CTL: If a transition occurs beginning from O progressing to a state fulfilling the formula CTL.

AF CTL: If each transition beginning from O progressing to a state fulfilling the formula CTL.

EF CTL: If a path occurs beginning from O that progresses to a state fulfilling the formula CTL.

AF CTL: If each path commencing from O progressing to a state fulfilling the formula CTL.

EG CTL: If a path starting from O that includes only those states fulfilling the formula CTL.

AG CTL: If each infinite path that begins from O includes those states fulfilling the formula CTL.

E CTL1 U CTL2: If a path occurs beginning from O that includes a state s fulfilling the formula CTL2 and each state earlier than s' in the path fulfills the formula CTL1.

A CTL1 U CTL2: If each path beginning from O includes a state s fulfilling the formula CTL2 and each state earlier than s' in the path fulfills the formula CTL 1.

3.2.2 Hybrid Modeling using Time Delays

Hybrid models permit joint representation for both discrete and continuous models. They have been effectively utilized within the most recent decades, specifically for the validation and verification of real-time and embedded systems. Generally, a hybrid model involves a clock along with a location (discrete). It also consists of continuous transition that represents the time lapsed at that location. During evolution of a gene/protein c, its expression level moves from abstract level x to $x+1$ after a certain time delay d_c^+. Similarly, during its inhibition its abstract level changes from $x+1$ to x after the time delay d_c^-. A sufficient change in the concentration of a protein occurs after the mentioned time delay periods. Figure 3.1 represents the activation and inhibition of a gene/ protein with reference to time delays [30].Hybrid models permit joint representation for both discrete and continuous models. They have been effectively utilized within the most recent decades, specifically for the validation and verification of real-time and embedded systems. Generally, a hybrid model involves a clock along with a location (discrete). It also consists of continuous transition that represents the time lapsed at that location. During evolution of a gene/protein c, its expression level moves from abstract level x to $x+1$ after a certain time delay d_c^+. Similarly, during its inhibition its abstract

level changes from *x+1* to x after the time delay d_c^-. A sufficient change in the concentration of a protein occurs after the mentioned time delay periods. Figure 3.1 represents the activation and inhibition of a gene/ protein with reference to time delays [31].

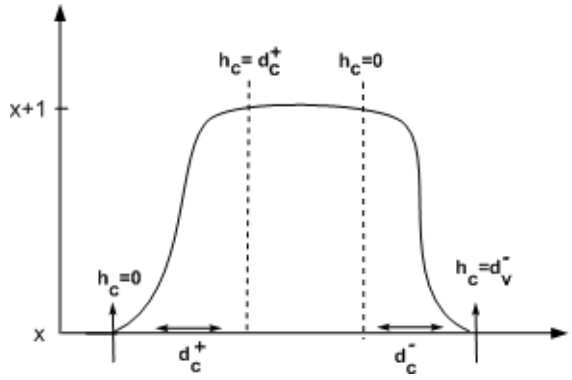

Figure 3.1: Continuous evolution. Actual evolution related to expression of a gene. [28].

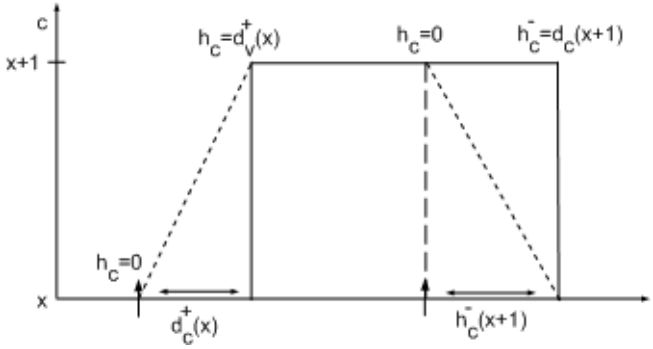

Figure 3.2: Discrete Evolution. Discrete approximation of the actual evolution [28].

Clocks are continuous type variables utilized within timed automaton models [32], which belong to a class of hybrid automata [33]. Each protein is connected with a clock variable h that synchronously evolves with time (rate of clock is equal to 1). These clock delays reflect the attributes of continuous dynamics inside the accessible discrete formalism [30]. The time measured by the clock variable h between two levels is known as the delay between these levels. The starting valuation of the clock variable is set as zero and when the estimation of this clock variable is equivalent to delay time d^+ or d^-, the move between two

levels happens. The delay d^+ or d^- represents time taken from x to x+1 (positive delay) or x+1 to x (negative delay), respectively (figure 3.1 and 3.2). The speed by which the clock variable h advances is demonstrated by the derivative $d_c/d_t = \xi$ (rate), where rate lies in the set {0,1,-1}. This portrays the advancement of the associated variable, which demonstrates and represents evolution level of a protein [30, 34]. We hence get a hybrid model that is suitable to represent the continuous and discrete dynamics of the frameworks that we considered. In this current research, unvalued parameters are considered as delays which lead to the definition of parametric Bio Linear Hybrid Automata (Bio-LHA) for the representation of the E2F1 and miR-223 related BRN, as discussed in the following section.

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representation of the E2F1 and miR-223 related BRN, as discussed in the following section.

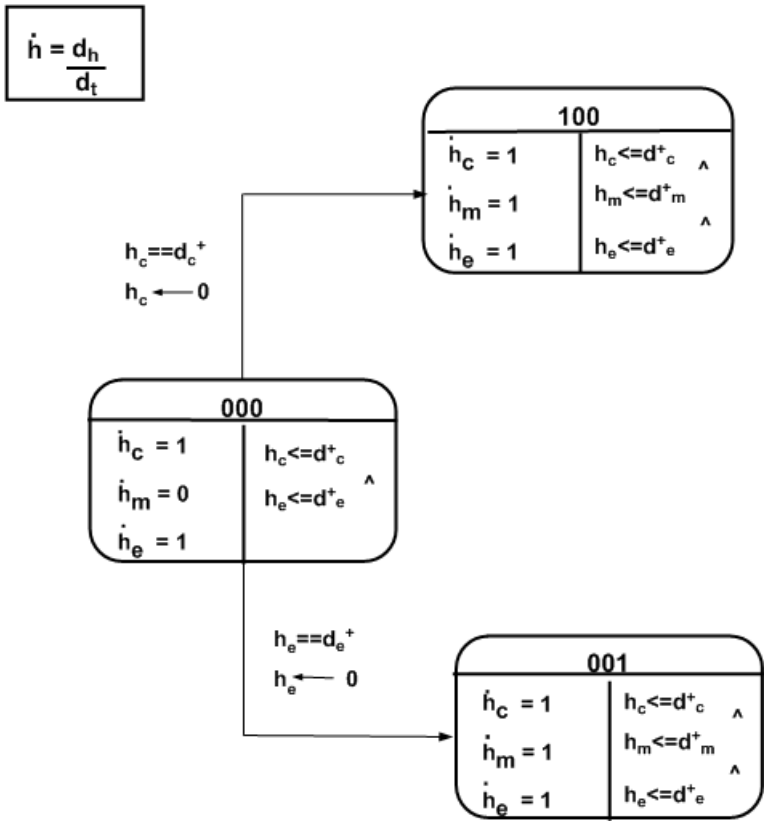

Figure 3.3: Patial view of Bio-LHA of the AML associated BRN. States are represented by 100, 000, and 001 consisting of three entities i.e. C/EBPα(c), miR-223(m) and E2F1(e)

"Let the set of constraints $C^{=}(X, P)$, $C^{\leq}(X, P)$, and $C^{\geq}(X, P)$ for $=$, $\leq$, and $\geq$, respectively, where the real valued variables are given X and parameters by P."Formally, the Bio-LHA is then defined as,

Definition 8 (Parametric Bio-LHA). *"Parametric Bio-LHA $\mathbb{B}$ is represented as tuple (L, l_0, X, P, E, Inv, Dif) where*

- *L is a finite set of locations,*
- *$l_0 \in$ is the initial location,*
- *P is a finite set of parameters (delays),*

- *X is a finite set of real-valued variable (clocks),*
- *$E \subseteq L \times C=(X,P) \times 2^X \times L$ represents the finite set of edges, e=(l,g,R,l')$\in E$ represents an edge from location l to location l' with the guard g on the transition and reset set $R \subseteq X$ i.e. set of clocks in guard g is a subset of R.*
- *Inv: $L \rightarrow C^{\leq}(X,P) \bigcup C^{\geq}(X,P)$ assigns an invariant to any location,*
- *Dif: $L \times X \rightarrow$ {-1,0,1} maps each pair (l, h) to an evolution rate.*

The Transition System related semantics of the parametric Bio-LHA is given below according to the time domain T, where $T^* = T/\{0\}$." [29]

Definition 9 (Semantics of Bio-LHA). *"Let γ be a valuation for the parameters P and υ represents the values of clocks in a location. The ($\mathbb{T}$, γ)-semantics of a parametric Bio-LHA H = (L, l_0, X, P, E, Inv, Dif) is defined as a timed transition system $\mathbb{S}_H$ = (S, s_0,T,$\rightarrow$) where:*

1. $\mathbb{S} = \{(l, \upsilon) \mid l \in L$ *and* $\upsilon \vDash Inv(l)\}$.
2. s_0 *is the initial state and*
3. *the relation* $\rightarrow \subseteq \mathbb{S} \times \mathbb{T} \times \mathbb{S}$ *is defined for* $t \in \mathbb{T}$ *as* :

- **discrete transitions:** *$(l, \upsilon) \xrightarrow{0} (l', \upsilon')$ iff $\exists$ (l,g,R,l') $\in E$ such that $g(\upsilon)$= true, $\exists'(h)= 0$ if $h \in R$ and $\upsilon'(h)$ if $h \notin R$*
- **continuous transitions:** *For $t \in \mathbb{T}^*$, $(l, \upsilon) \rightarrow (l', \upsilon')$ iff $l' = l$, $(\upsilon'(h)= \upsilon(h)+ Dif(l,h) \times t$, and for every $t' \in [0,t]$, $(\upsilon (h)+ Dif(l,h) \times t') \vDash inv(l)$ ". [29]*

The partial view of Bio-LHA of the AML associated BRN is represented in figure 3.3 where dh represents the rate of clock and the inequalities represent the location invariant e.g. $h_c \leqslant d_c^+$ briefs that clock associated with C/EBPα should be strictly less than or equal to activation delay of C/EBPα.

Utilizing the semantics of the Bio-LHA by Ahmad et al [30] we defined the temporal state space and the invariance kernel set which has been stated below.

Denition 10 (Temporal Zone). *"Temporal zone is defined as a zone where time elapses until a discrete transition between states happens."* [28]

Definition 11 (Temporal State Space). *"The temporal state of a BRN is made out of the complete set of temporal zones inferred from the discrete model of the said BRN."* [28]

Definition 12. (Invariance Kernel). *"A trajectory $\psi(t)$ is viable in set $\mathbb{S}$ if $\psi(t) \in \mathbb{S}$ for all $t \geq 0$. The subset K of $\mathbb{S}$ is said to be invariant if for any point $p \in K$ there exist a minimum one trajectory starting in p, and every trajectory starting in p is viable in K. Given a set $\mathbb{S}$, its largest invariant subset is called the invariance kernel of $\mathbb{S}$. "* [28]

Chapter 4

Results and Discussion

4.1 Selection of Logical parameters

The BRN was constructed on the basis of methodology defined in the previous section. The edges defined in figure 4.1 represent the following behavior of TFs related to AML.

- According to the René Thomas multivalued approach sixteen possible combinations of the BRN were possible (on the basis of principle that each node can have a threshold value equal to its outgoing degree). Although the boolean approach was enough to discuss about the steady stable states of the TFs, but eventually it didn't lead towards the cyclic behaviors which are quite important for this particular regulatory network [35].

- The different number of BRNs according to possible threshold values were then given to SMBioNet for the deduction of the parameters, the source code of which is given in Appendix. For convenience, the CTLs for sixteen different BRNs are divided into four cases. Sections are made on the basis of maximum threshold values of C/EBPα and E2F1.

Case 1

$$(C{=}0 \wedge M{=}0 \wedge E{=}0) \rightarrow AX(EF(C{=}0 \wedge M{=}0 \wedge E{=}0)) \tag{4.1}$$

$$(C{=}0 \wedge M{=}0 \wedge E{=}0) \rightarrow E\ F\ (A\ G(C{=}2 \wedge M{=}1 \wedge E{=}0)) \tag{4.2}$$

$$(C{=}0 \wedge M{=}0 \wedge E{=}0) \rightarrow E\ F\ (A\ G(C{=}0 \wedge M{=}0 \wedge E{=}2)) \tag{4.3}$$

Case 2

$$(C{=}0 \wedge M{=}0 \wedge E{=}0) \rightarrow E\ F\ (A\ G(C{=}2 \wedge M{=}1 \wedge E{=}0)) \tag{4.4}$$

$$(C{=}0 \wedge M{=}0 \wedge E{=}0) \rightarrow E\ F\ (A\ G(C{=}0 \wedge M{=}0 \wedge E{=}1)) \tag{4.5}$$

Case 3

$$(C{=}0 \wedge M{=}0 \wedge E{=}0) \rightarrow E\ F\ (A\ G(C{=}1 \wedge M{=}1 \wedge E{=}0)) \tag{4.6}$$

$$(C{=}0 \wedge M{=}0 \wedge E{=}0) \rightarrow E\ F\ (A\ G(C{=}0 \wedge M{=}0 \wedge E{=}2)) \tag{4.7}$$

Case 4:

$$(C{=}0 \wedge M{=}0 \wedge E{=}0) \rightarrow E\ F\ (A\ G(C{=}1 \wedge M{=}1 \wedge E{=}0)) \tag{4.8}$$

$$(C{=}0 \wedge M{=}0 \wedge E{=}0) \rightarrow E\ F\ (A\ G(C{=}0 \wedge M{=}0 \wedge E{=}1)) \tag{4.9}$$

In the above mentioned cases 'C' represents the transcription factor C/EBPα, 'M' represents *miRNA-223*, and 'E' represents another transcription factor E2F1. The above mentioned CTLs are used to find parameters of such BRNs in which the maximum threshold values of C/EBPα and E2F1 are taken up till 2. The initial state represents the state where all entities are at threshold level '0'. The first CTL formula implements the condition that all successor states from the starting state (0,0,0) are constantly ready to evolve and will eventually end at the initial state (0,0,0) (cyclic behavior). The second CTL formula suggests that from the initial state there exist a path in future in which all paths starting from the given initial location contains only a state satisfying the given CTL formula. In simpler words there exist a path from the initial state (0,0,0) that will lead to a state where miR-223 and C/EBPα are at maximum threshold level 1 and 2, respectively. This CTL represents a biological phenomenon that during normal granulopoiesis C/EBPα up regulates and transactivates miR-223 promoter, which thus prompts E2F1 suppression and restraints cell-cycle movement bringing forward myeloid differentiation. This explains the fact that when C/EBPα is at its maximum threshold 2 it also activates miR-223 at its maximum threshold level 1 which in return deactivates E2F1 with threshold level -1. The third CTL represents that from the initial state there exist a path that will lead towards the diseased condition (0,0,2). Biologically, it is said that targeted interruption in C/EBPα

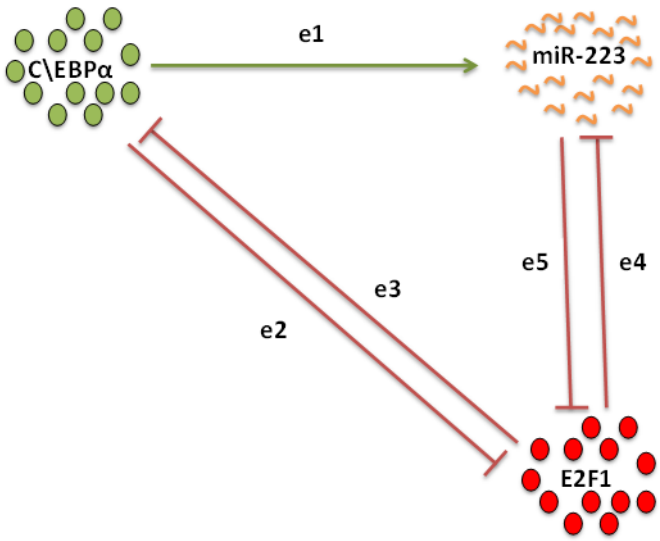

Figure 4.1: AML associated BRN. Auto regulatory feedback loop between C/EBPα, miR-223 and E2F1. Whereas edges are represented by e1, e2, e3, e4 and e5 respectively. Arrows indicate the direction of activation/inhibition

required for E2F1 binding brings about block of granulopoiesis in mice [36]. Study reveals that, mice convey a germline transformation that deregulates C/EBPα linked E2F suppression, and develop AML. Interestingly, E2F1 has the capacity to repress granulopoiesis and forces myeloid cell-cycle movement. Cases 2 and 3 also represent the above mentioned phenomenon but with different threshold values of C/EBPα and E2F1. There is a bit difference in case 4 i.e. the CTL related to cyclic behavior of initial condition is not included because there does not exist a cyclic trajectory when the BRN in figure 4.1 is made with the help of Boolean formalism proposed by René Thomas.

Table 4.1: Name of BRN models according to their CTL conditions.

CTL Cases	**Threshold of BRNs in the order of e1, e2, e3, e4 and e5**
Case 1	12221, 21211, 12121, 12211, 21121, 22211, 22121, 21221,22221
Case 2	21111,12111, 22111
Case 3	11121, 11211, 11221
Case 4	11111

4.2 Results Generated from SMBioNet

The parameters generated by SMBioNet show significant expression levels according to the set of resources. When any activator is mentioned in the set, it represents its presence and in case of inhibitor it represent its absence or vice versa e.g. K_miR-223 {C/EBPα, E2F1} = 1 suggests the presence of its activator C/EBPα and absence of its inhibitor E2F1 leads to evolution of miR-223 towards its maximum threshold level i.e. 1. Rest of the parameters and their selected values are mentioned in table 4.2 according to different cases.

Table 4.2: Possible parameter values for the selected models.

Parameters	**Resources**	**Selected Values for Case 1**	**Selected Values for Case 2**	**Selected Values for Case 3**	**Selected Values for Case 4**
K_C/EBPα	ϕ	0	0	0	0
	{E2F1}	2	2	1	1
K_miR-223	ϕ	0	0	0	0
	{C/EBPα}	1	1	1	1
	{E2F1}	0	0	0	0
	{E2F1, C/EBPα}	1	1	1	1
K_E2F1	ϕ	0	0	0	0
	{C/EBPα}	0	0	0	0
	{miR-223}	0	0	0	0
	{C/EBPα, miR-223}	2	1	2	1

4.3 Qualitative Analysis of State Graph

The selected parameters mentioned in table 4.2 were used to model the BRN using GENOTECH. The resultant state graphs of the above mentioned four cases comprised of 18 states (for case 1), 12 states (for cases 2 and 3) and 8 states (for case 4). In each case, two deadlocks were observed.

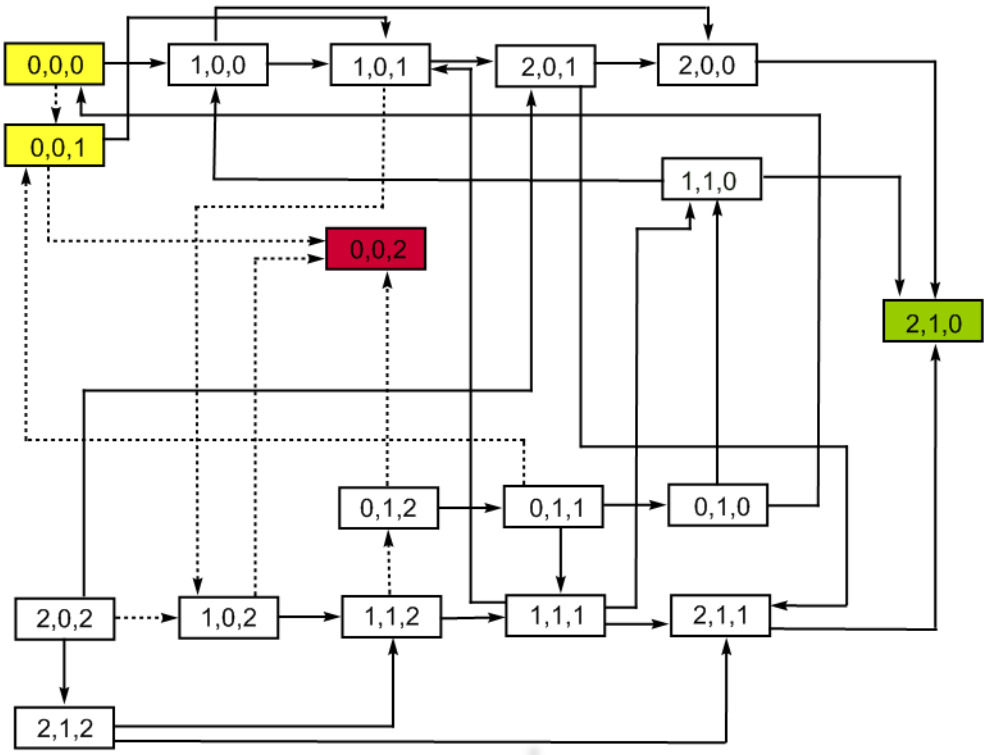

Figure 4.2: Normal and divergent trajectories (a). State Graph corresponding to Case 1. Each block represents an entity and the values inside them indicate qualitative levels of TFs according to the order (C/EBPα, miR-223, E2F1) Two steady stable states were observed. The first deadlock (2,1,0) indicates the overexpression of C/EBPα and miR-223 and low expression of E2F1 that lead towards blockage of S phase during cell cycle resulting in granulopoiesis. The second deadlock (0,0,2) indicates the high expression of E2F1 which results in myeloid cell proliferation leading towards AML. Dotted lines indicate the bifurcating states that lead towards deadlock (0,0,2)

- First deadlock (2,1,0) or (1,1,0), depicted the same phenomenon i.e. when C/EBPα is overexpressed in normal stem cells, it regulates the expression of miR-223 which in return deactivates the transcriptional activity of transcription factor E2F1 and facilitates the process of normal granulocytic differentiation [19, 37].

- Second deadlock (0,0,2) or (0,0,1) signifies the cellular proliferation leading to AML. These phenomena suggest that when a chromosomal translocation occurs e.g. AML1/ETO miR-223 is deregulated which leads towards the aggregation of E2F1. The overexpressed E2F1 binds at the transcriptional/promoter site of miR-223 (instead of allowing C/EBPα to bind at

promoter site of miR-223) thus leading towards transcriptional block. Lack of production of miR-223 is regulated by a negative feedback loop leading towards uncontrolled cell proliferation ehich is the main cause of AML.

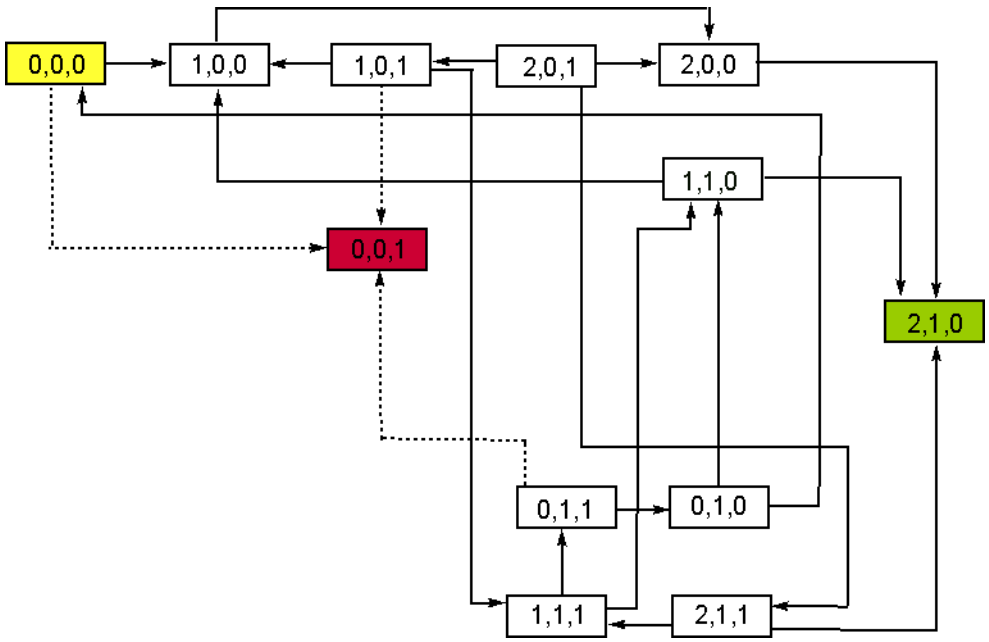

Figure 4.3: Normal and divergent trajectories (b). State Graph corresponding to Case 2 and 3. Dotted lines indicate the bifurcating states.

Amongst 16 BRNs, cyclic behavior was observed in only four of them i.e. BRN 12221 , BRN 12121, BRN 12211 and BRN 12111. It is very important to observe cyclic behaviour as they lead towards homeostasis. The human body deals with large number of exceptionally perplexing collaborations to keep up offset or maintain feedbacks to work inside a typical range. These communications inside the body encourage important steady changes related to physical and chemical working. This procedure is fundamental to the survival of the humans. Similarly, few transcription factors or proteins play crucial role in maintaining homeostasis referring to any specific phenomena e.g. in case of granulopoiesis, expression levels of C/EBPα and E2F1 play important role in maintaining homeostasis which when disturbed may lead to AML. The number of cycles also show the evidence that granulopoiesis or proliferation – both of them have options to evolve in response

to changes in transcription factors at any given moment. States graphs of the BRNs showing the cyclic behavior are mentioned in the figures 4.2 and 4.3. The state diagram, demonstrating stable states, shows up in another window with it set of instructions to discover cycles, paths, and neighboring states (see Figure 10). GENOTECH, likewise gives a choice to save the state graph in DOT format [38] for visualization in Graphviz software [39].

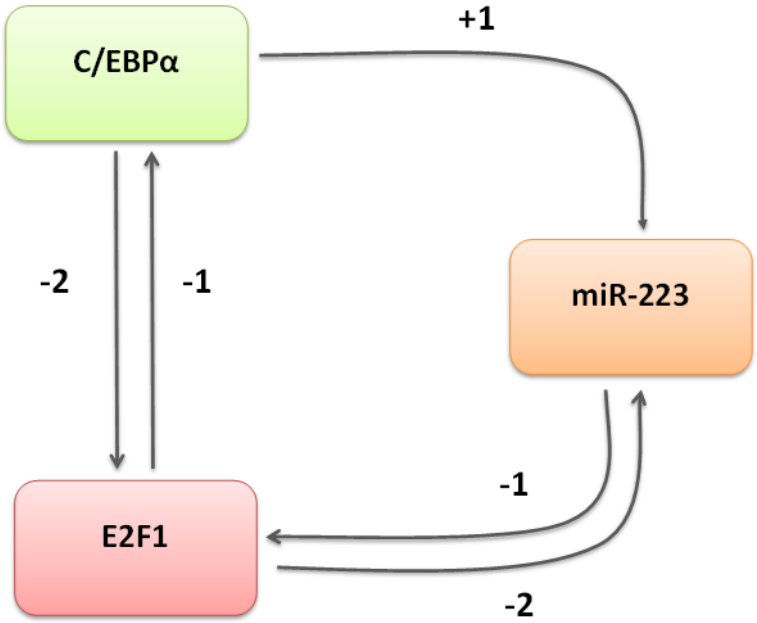

Figure 4.4: AML associated BRN along with threshold values. One of the AML associated auto regulatory negative feedback loop based on multivalued formalism from which cycles were observed and further Hytech results were deduced from it. Furthermore, it suggests that multivalued AML associated BRN was better than boolean formalism as it also deals with the cyclic trajectories which were not observed in BRN with boolean thresholds.

4.4 Refinement of Cycles Using Time Delays

As defined earlier refinement of cycles using time delays is done by hybrid modeling. HyTech is a Linux-based symbolic model checker for linear hybrid automata of embedded and real-time systems. HyTech computes the constraints of the pa-

rameters to find good values such that the system satisfies a given property. It is used to get the constraints of BRN that do predictions about production and degradation time of proteins of the pathway [40].

The selected cycle (on the basis of convergence to produce an Invariance Kernel) were then converted into BIO-LHA. The invariants, rates, guards and other parameters were assigned as per the detailed modeling description given in section 3, with the HyTech source code provided in Appendix.

Table 4.3: Cycles Obtained from GENOTECH

Number of Cycles	**Cyclic Behaviour of Entities (in Order of C/EBPα, miR-223 and E2F1)**
Cycle 1	[(0,0,0), (1,0,0), (1,0,1), (1,0,2), (1,1,2), (0,1,2), (0,1,1), (0,1,0), (0,0,0)]
Cycle 2	[(0,0,0), (1,0,0), (1,0,1), (1,0,2), (1,1,2), (1,1,1), (0,1,1), (0,1,0), (0,0,0)]
Cycle 3	[(0,0,0), (1,0,0), (1,0,1), (1,1,1), (0,1,1), (0,1,0), (0,0,0)]

"We define the full period (denoted π(u))as the sum of all delays for a gene u to pass sequentially and successively, once through each of all its existance [41]."

The BRNs under study generated three cyclic trajectories using HyTech having some common overlapping states. The above mentioned cycles in table 4.3 were modeled in HyTech. The delay constraints generated by model checker are composed of equalities and inequalities which determine the stability or instability of the cycle. The most important delay constraints are discussed below:

1. $2\delta^{+}_{E2F1_0} + \delta^{+}_{C/EBP\alpha_0} + \mid \delta^{-}_{C/EBP\alpha_1} \mid = \delta^{+}_{miR-223_0} + \mid \delta^{-}_{miR-223_1} \mid$

   We can deduce important relation from the above equation i.e.

   - π *(C/EBP* α *)* $\leqslant$ π *(miR-223)*

   Thus the BRN in which the path leading towards the granulopoiesis is involved during the blockage of S phase is possible when C/EBP α full period is less than that of a full period of miR-223.

This proves the fact that human granulopoiesis is controlled by a negative feedback loop including miR-223 and two transcriptional variables, E2F1 and C/EBPα. These two TFs compete for binding with the miR-223 promoter: E2F1 keeps miR-223 at low levels, though its substitution by C/EBPα, upregulates miR-223 transcription. The opposition by C/EBPα and the granulocytic separation are supported by a negative feedback loop in which miR-223 suppresses E2F1 translation [17, 42].

The second very significant constraint implies that

2. $\delta^{+}_{C/EBP\alpha_0} = |\delta^{-}_{E2F1_1}|$

   This illustrates that activation delay of C/EBPα is equal to the negative delay of E2F1. This constraint predicts that the rate of activation of C/EBPα is equal to the rate of inhibition of E2F1 during sustained homeostatsis (characterized by invariance kernel) This property is hard to verify by *in vitro* means and generally it can be biologically proven with the fact that C/EBPα strictly controls cell-cycle regulation and is evolved during differentiation of myeloid cells. The development hindering movement of C/EBPα suppress tumorigenesis in myeloid cells and other different tissues. Several studies have investigated the system by which C/EBPα supresses proliferation at the G1-S phase through E2F system. It is also suggested that E2F1 binds to miR-223 promoter in the uninduced state and this coupling is diminished by C/EBP α interference. During granulopoiesis, C/EBP α binds and transactivates miR-223 promoter, which prompts E2F1 deregulation and hindrance of cell-cycle movement bringing about myeloid differentiation.

3. *L= π (miR-223)*

The above mentioned equation states that complete oscillation of miR-223 takes more time as compared to other four entities involved in the cycle. This can

also be seen from the relationship 1 (in case of C/EBPα) that period of miR-223 is greater. Furthermore it also shows that reactivity of AML is related to the the delay taken by miR-223 to complete its qualitative cycle.

Chapter 5

Conclusion

In conclusion, our study gives deeper insights about the computational proof related to different leukemic conditions created as a consequence of chromosomal translocations in E2F1 and miR-223 mediated regulatory pathway. Computational discrete modeling related to AML mediated pathway demonstrated that threshold levels of E2F1 carries out a vital role in myeloid cell proliferation by diminishing the role of C/EBPα in the the transcription of miR-223 promoter. Discrete modeling formalism of René Thomas followed by hybrid modeling is a well-known methodology utilized for demonstration and examination of BRNs. In this research, we predicted that the activity of E2F1 at different threshold levels is the turning point towards clearance or propagation of tumor. Our results verified that higher concentration of C/EBPα should be maintained in cells in order to deregulate E2F1 mediated feedback loop. The over expression of E2F1 favours myeloid cell proliferation which may further lead to inactivation of miR-223 resulting in AML. The important delay constraints explains that in order to maintain the homeostasis degradation rate of E2F1 must be kept equal to the activation rate of C/EBPα. However the violation of this constraint diverge the system towards the deadlock(unfavorable state). Thus these factors prove to be an appealing focus of new medication for AML patients. Pulikkan et.al. have re-

vealed that mutant form of C/EBPα fails to interact with E2F1 to induce miR-223 production. Similarly, several other studies also reveal that E2F1 is a potential target in designing drugs for AML patients. Our results verify these studies and also suggest that suppression of E2F1 would be beneficial. Accordingly, while designing medications against AML, the expression level of E2F1 must be down regulated by utilizing miR-223 or by planning such inhibitors for E2F1 proteins which keep them inactive. This would increase the activation rate of C/EBPα and helps in higher expression of miR-223. The present study helps us to comprehend the molecular system of E2F1 and miR-223 mediated pathway and to identify new targets for drug design. The above analysis urge that down regulation of E2F1 can help in maintaining homoeostasis by regulating the expression of C/EBPα , which further regulates miR-223 transcription. The present study, affirms that focusing on E2F1 in miR-223 mediated pathway can be helpful and prognostic marker against AML. Previously, it was accounted that C/EBPα may be focused for medications in AML, since C/EBPα is likewise included in other diverse cell activities consequently we recommend that E2F1 may be a superior focus as compared with C/EBPα and miR-223. These findings recommend that treatments against AML should target E2F1 i.e. inhibitor should be intended to suppress E2F1 activity.

Bibliography

[1] Webmd.com. Cancer center: Types, symptoms, causes, tests, and treatments, including chemo and radiation, 2015.

[2] Nlm.nih.gov. Cancer: Medlineplus, 2015.

[3] Abta.org. Types of tumors — american brain tumor association, 2015.

[4] H.H. Naumann, F. Martin, H. Scherer, and K. Schorn. *Differential Diagnosis in Otorhinolaryngology: Symptoms, Syndromes, and Interdisciplinary Issues*. G. Thiem Verlag, 1993.

[5] Judah Folkman. Tumor angiogenesis: therapeutic implications. *New England Journal of Medicine*, (285):1182–6, 1971.

[6] Wallace H Clark, David E Elder, Dupont Guerry, Martin N Epstein, Mark H Greene, and Marie Van Horn. A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma. *Human pathology*, 15(12):1147–1165, 1984.

[7] Cancer.org. American cancer society expert voices blog, 2013.

[8] Medical Today. What is cancer? what causes cancer?, 2015.

[9] Hans Albertsen, Rakesh Anand, Mary Carlson, Joanna Groden, Philip J Hedge, Geoff Joslyn, Kenneth Kinzler, Alexander F Markham, Yusuke Naka-

mura, Andrew Thliveris, et al. Diagnosis, therapy cancer, October 4 1994. US Patent 5,352,775.

[10] Phyllis N Butow, John N Kazemi, Linda J Beeney, Anne-Marie Griffin, Stewart M Dunn, and Martin HN Tattersall. When the diagnosis is cancer: patient communication experiences and preferences. *Cancer*, 77(12):2630–2637, 1996.

[11] American Cancer Society. *Cancer facts & figures*. The Society, 2008.

[12] Cancercenter.com. Types of leukemia: 4 primary types — ctca, 2015.

[13] Barbara Deschler and Michael Lübbert. Acute myeloid leukemia: epidemiology and etiology. *Cancer*, 107(9):2099–2107, 2006.

[14] Bob Lowenberg, James R Downing, and Alan Burnett. Acute myeloid leukemia. *New England Journal of Medicine*, 341(14):1051–1062, 1999.

[15] Cancer.org. What are the key statistics about acute myeloid leukemia? — american cancer society.

[16] Elihu Estey and Hartmut Döhner. Acute myeloid leukaemia. *The Lancet*, 368(9550):1894–1907, 2006.

[17] Claus Nerlov. The c/ebp family of transcription factors: a paradigm for interaction between gene expression and proliferation control. *Trends in cell biology*, 17(7):318–324, 2007.

[18] Jun Lu, Gad Getz, Eric A Miska, Ezequiel Alvarez-Saavedra, Justin Lamb, David Peck, Alejandro Sweet-Cordero, Benjamin L Ebert, Raymond H Mak, Adolfo A Ferrando, et al. Microrna expression profiles classify human cancers. *nature*, 435(7043):834–838, 2005.

[19] Jonathan B Johnnidis, Marian H Harris, Robert T Wheeler, Sandra Stehling-Sun, Michael H Lam, Oktay Kirak, Thijn R Brummelkamp, Mark D Fleming,

and Fernando D Camargo. Regulation of progenitor cell proliferation and granulocyte function by microrna-223. *Nature*, 451(7182):1125–1129, 2008.

[20] John A Pulikkan, Viola Dengler, Philomina S Peramangalam, Abdul A Peer Zada, Carsten Müller-Tidow, Stefan K Bohlander, Daniel G Tenen, and Gerhard Behre. Cell-cycle regulator e2f1 and microrna-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. *Blood*, 115(9):1768–1778, 2010.

[21] William CS Cho. Oncomirs: the discovery and progress of micrornas in cancers. *Molecular cancer*, 6(1):60, 2007.

[22] M Haneklaus, M Gerlic, LAJ O'Neill, and SL Masters. mir-223: infection, inflammation and cancer. *Journal of internal medicine*, 274(3):215–226, 2013.

[23] Types leukaemia. Types of treatment for acute myeloid leukaemia — cancer research uk, 2015.

[24] Antonio Majdandzic, Boris Podobnik, Sergey V Buldyrev, Dror Y Kenett, Shlomo Havlin, and H Eugene Stanley. Spontaneous recovery in dynamical networks. *Nature Physics*, 10(1):34–38, 2014.

[25] Sven Erik Jørgensen. Structural dynamic model. *Ecological Modelling*, 31(1):1–9, 1986.

[26] Gilles Bernot, Franck Cassez, Jean-Paul Comet, Franck Delaplace, Céline Müller, and Olivier Roux. Semantics of biological regulatory networks. *Electronic Notes in Theoretical Computer Science*, 180(3):3–14, 2007.

[27] Hannes Klarner, Adam Streck, David Šafránek, Juraj Kolčák, and Heike Siebert. Parameter identification and model ranking of thomas networks. In *Proceedings of the 10th International Conference on Computational Meth-*

ods in Systems Biology, CMSB'12, pages 207–226, Berlin, Heidelberg, 2012. Springer-Verlag.

[28] Jamil Ahmad and Olivier Roux. Invariance kernel of biological regulatory networks. *International journal of data mining and bioinformatics*, 4(5):553–570, 2010.

[29] Babar Aslam, Jamil Ahmad, Amjad Ali, Rehan Zafar Paracha, Samar Hayat Khan Tareen, Umar Niazi, and Tariq Saeed. On the modelling and analysis of the regulatory network of dengue virus pathogenesis and clearance. *Computational biology and chemistry*, 53:277–291, 2014.

[30] Jamil Ahmad, Gilles Bernot, J-P Comet, Didier Lime, and Olivier Roux. Hybrid modelling and dynamical analysis of gene regulatory networks with delays. *ComPlexUs*, 3(4):231–251, 2007.

[31] Jamil Ahmad, Gilles Bernot, Jean-Paul Comet, Didier Lime, and Olivier Roux. Hybrid modelling and dynamical analysis of gene regulatory networks with delays. *ComPlexUs*, 3(4):231–251, 2006.

[32] Rajeev Alur and David L Dill. A theory of timed automata. *Theoretical computer science*, 126(2):183–235, 1994.

[33] Thomas A Henzinger. *The theory of hybrid automata*. Springer, 2000.

[34] Jamil Ahmad, Olivier Roux, Gilles Bernot, and Jean-Paul Comet. Analysing formal models of genetic regulatory networks with delays. *International journal of bioinformatics research and applications*, 4(3):240–262, 2008.

[35] Gilles Bernot, Jean-Paul Comet, Adrien Richard, and Janine Guespin. Application of formal methods to biological regulatory networks: extending thomas asynchronous logical approach with temporal logic. *Journal of theoretical biology*, 229(3):339–347, 2004.

[36] Guo-Li Wang and Nikolai A Timchenko. Dephosphorylated c/ebpα accelerates cell proliferation through sequestering retinoblastoma protein. *Molecular and cellular biology*, 25(4):1325–1338, 2005.

[37] Francesco Fazi, Alessandro Rosa, Alessandro Fatica, Vania Gelmetti, Maria Laura De Marchis, Clara Nervi, and Irene Bozzoni. A minicircuitry comprised of microrna-223 and transcription factors nfi-a and c/ebpα regulates human granulopoiesis. *Cell*, 123(5):819–831, 2005.

[38] Code.google.com. Genotech.zip - genotech - plos one - genotech software - genotech is a tool for the modeling of biological regulatory networks - google project hosting, 2012.

[39] Graphviz.org. Graphviz — graphviz - graph visualization software, 2015.

[40] Thomas A Henzinger, Pei-Hsin Ho, and Howard Wong-Toi. Hytech: A model checker for hybrid systems. In *Computer aided verification*, pages 460–463. Springer, 1997.

[41] Jamil Ahmad, Jérémie Bourdon, Damien Eveillard, Jonathan Fromentin, Olivier Roux, and Christine Sinoquet. Temporal constraints of a gene regulatory network: Refining a qualitative simulation. *Biosystems*, 98(3):149–159, 2009.

[42] L Vian, M Di Carlo, E Pelosi, F Fazi, S Santoro, AM Cerio, A Boe, V Rotilio, M Billi, S Racanicchi, et al. Transcriptional fine-tuning of microrna-223 levels directs lineage choice of human hematopoietic progenitors. *Cell Death & Differentiation*, 21(2):290–301, 2013.

Appendix

Appendix A

SMBioNet source files

Input file of AML Associated BRN

```
Generelized SMBioNet file

VAR
c=0 2;
m=0 1;
e=0 1;
REG
c [c>=1]=> m;
c [c<2]=> e;
m [m<1]=> e;
e [e<1]=> c;
e [e<1]=> m;
CTL
#((c=0&m=0&e=0)->AX(EF(c=0&m=0&e=0)))
#&
((c=0&m=0&e=0)->EF(AG(c=2&m=1&e=0)))
&
((c=0&m=0&e=0)->EF(AG(c=0&m=0&e=1)))
```

Output file of AML Associated BRN

```
# MODEL 1

# K_c = 0
# K_c+e = 2
```

```
# K_m = 0
# K_m+c = 0
# K_m+e = 0
# K_m+c+e = 1

# K_e = 0
# K_e+c = 0
# K_e+m = 0
# K_e+c+m = 1

# MODEL 2

# K_c = 0
# K_c+e = 2

# K_m = 0
# K_m+c = 1
# K_m+e = 0
# K_m+c+e = 1

# K_e = 0
# K_e+c = 0
# K_e+m = 0
# K_e+c+m = 1

# MODEL 3

# K_c = 0
# K_c+e = 2

# K_m = 0
# K_m+c = 0
# K_m+e = 1
# K_m+c+e = 1

# K_e = 0
# K_e+c = 0
# K_e+m = 0
# K_e+c+m = 1

# MODEL 4
```

```
# K_c = 0
# K_c+e = 2

# K_m = 0
# K_m+c = 1
# K_m+e = 1
# K_m+c+e = 1

# K_e = 0
# K_e+c = 0
# K_e+m = 0
# K_e+c+m = 1
```

Appendix B

Invariance Kernels

Table 1: The cycle and invariance kernel corresponding to the BRN 12111.

Cycle:	$000 \to 100 \to 101 \to 102 \to 112 \to 111 \to 011 \to 010 \to 000$
	Invariance Kernel (conjuncts of I–IV)
I.	$\pi(CEBPA_{0,1}) + 2\delta^+_{E2F1_0} = \pi(MIR223_{0,1})$
II.	$\lvert\delta^-_{E2F1_1}\rvert = \pi(CEBPA_{0,1}) + \delta^+_{E2F1_0}$
III.	$\delta^+_{E2F1_0} \leq \delta^+_{MIR223_0}$
IV.	$\delta^+_{MIR223_0} \leq 2\delta^+_{E2F1_0} + \lvert\delta^-_{CEBPA_1}\rvert$

Table 2: The first cycle and invariance kernel corresponding to the BRN 1212.

Cycle:	$000 \to 100 \to 101 \to 102 \to 112 \to 012 \to 011 \to 010 \to 000$
	Invariance Kernel (conjuncts of I–V)
I.	$\delta^+_{CEBPA_0} + \delta^+_{E2F1_0} + \pi(E2F1_{1,2}) = \pi(MIR223_{0,1})$
II.	$\delta^+_{CEBPA_0} = \lvert\delta^-_{E2F1_1}\rvert$
III.	$\delta^+_{E2F1_0} + \delta^+_{E2F1_1} \leq \delta^+_{MIR223_0}$
IV.	$\delta^+_{MIR223_0} \leq 2\delta^+_{E2F1_0} + \lvert\delta^-_{CEBPA_1}\rvert$
V.	$\delta^+_{E2F1_0} + \lvert\delta^-_{CEBPA_1}\rvert \leq \pi(E2F1_{1,2})$

Table 3: The second cycle and invariance kernel corresponding to the BRN 1212.

Cycle:	$000 \to 100 \to 101 \to 102 \to 112 \to 111 \to 011 \to 010 \to 000$
Invariance Kernel (conjuncts of I–V)	
I.	$\pi(E2F1_{1,2}) + \lvert\delta^-_{E2F1_1}\rvert = \pi(CEBPA_{0,1}) + \delta^+_{E2F1_0}$
II.	$\pi(CEBPA_{0,1}) + 2\delta^+_{E2F1_0} = \pi(MIR223_{0,1})$
III.	$\delta^+_{E2F1_0} + \delta^+_{E2F1_1} \leq \delta^+_{MIR223_0}$
IV.	$\delta^+_{MIR223_0} + \lvert\delta^-_{E2F1_1}\rvert \leq \pi(CEBPA_{0,1}) + 2\delta^+_{E2F1_0}$
V.	$\delta^+_{CEBPA_0} \leq \lvert\delta^-_{E2F1_1}\rvert$

Appendix C

HyTech Source Files

```
1st Cycle 12111



 -- gene No0 = CEBPA


 -- gene No1 = MIR223


 -- gene No2 = E2F1
 var
 dpCEBPA0,dpMIR2230,dpE2F10,dnMIR2231,dnE2F11,dpCEBPA1,dnCEBPA1,dnCEBPA2: parameter;
 hCEBPA,hMIR223,hE2F1,h :analog;
 k,n,l: discrete;
 automaton auto
synclabs: ;
 initially loc_000;
 -- for the configuration 0,0,0
 loc loc_000: while hCEBPA <=  dpCEBPA0   wait {dhCEBPA=1,dhMIR223=1,dhE2F1=1,dh=1}
 when hCEBPA=dpCEBPA0 do {hCEBPA'=0, k'=k+1, l'=l+h, h'=0} goto loc_100;
 -- for the configuration 0,1,0
 loc loc_010: while hCEBPA <=  dpCEBPA0 & hMIR223 >=  dnMIR2231  wait {dhCEBPA=1,dhMIR223=-1,dhE2F1=1,dh=1}
 when hMIR223=dnMIR2231 do {hMIR223'=0, k'=k+1, l'=l+h, h'=0} goto loc_000;
 -- for the configuration 0,1,1
 loc loc_011: while hE2F1 >=  dnE2F11  wait {dhCEBPA=1,dhMIR223=-1,dhE2F1=-1,dh=1}
 when hE2F1=dnE2F11 do {hE2F1'=0, k'=k+1, l'=l+h, h'=0} goto loc_010;
```

```
-- for the configuration 1,0,0
loc loc_100: while hE2F1 <=  dpE2F10  wait {dhCEBPA=1,dhMIR223=1,dhE2F1=1,dh=1}
when hE2F1=dpE2F10 do {hE2F1'=0, k'=k+1, l'=l+h, h'=0} goto loc_101;
-- for the configuration 1,0,1
loc loc_101: while hMIR223 <=  dpMIR2230  wait {dhCEBPA=-1,dhMIR223=1,dhE2F1=-1,dh=1}
when hMIR223=dpMIR2230 do {hMIR223'=0, k'=k+1, l'=l+h, h'=0} goto loc_111;
-- for the configuration 1,1,1
loc loc_111: while hCEBPA >=  dnCEBPA1   wait {dhCEBPA=-1,dhMIR223=-1,dhE2F1=-1,dh=1}
when hCEBPA=dnCEBPA1 do {hCEBPA'=0, k'=k+1, l'=l+h, h'=0} goto loc_011;
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_000 & hCEBPA>=0 & hMIR223>=0 & hE2F1=0 & hCEBPA <=  dpCEBPA0;
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 hCEBPA,hMIR223,hE2F1,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 hCEBPA,hMIR223,hE2F1,h in hull(pln_cyc_length & r_ini) endhide;
prints "============================================================";
prints "Length of a nested cycle is:";

print hide hCEBPA,hMIR223,hE2F1,h in hull(nes_cyc_length & r_ini) endhide;
prints "============================================================";
endif;
```

```
2nd Cycle 12121


 -- gene No0 = CEBPA


 -- gene No1 = MIR223


 -- gene No2 = E2F1


 var
 dpCEBPA0,dpMIR2230,dpE2F10,dnMIR2231,dnE2F11,dpCEBPA1,dnCEBPA1,dnCEBPA2: parameter;
 hCEBPA,hMIR223,hE2F1,h :analog;
 k,n,l: discrete;
 automaton auto
synclabs: ;
initially loc_000;
 -- for the configuration 0,0,0
loc loc_000: while hCEBPA <=  dpCEBPA0   wait {dhCEBPA=1,dhMIR223=1,dhE2F1=1,dh=1}
 when hCEBPA=dpCEBPA0 do {hCEBPA'=0, k'=k+1, l'=l+h, h'=0} goto loc_100;
 -- for the configuration 0,1,0
loc loc_010: while hCEBPA <=  dpCEBPA0 & hMIR223 >=  dnMIR2231  wait {dhCEBPA=1,dhMIR223=-1,dhE2F1=1,dh=1}
when hMIR223=dnMIR2231 do {hMIR223'=0, k'=k+1, l'=l+h, h'=0} goto loc_000;
 -- for the configuration 0,1,1
 loc loc_011: while hE2F1 >=  dnE2F11  wait {dhCEBPA=1,dhMIR223=-1,dhE2F1=-1,dh=1}
 when hE2F1=dnE2F11 do {hE2F1'=0, k'=k+1, l'=l+h, h'=0} goto loc_010;
 -- for the configuration 1,0,0
loc loc_100: while hE2F1 <=  dpE2F10  wait {dhCEBPA=1,dhMIR223=1,dhE2F1=1,dh=1}
when hE2F1=dpE2F10 do {hE2F1'=0, k'=k+1, l'=l+h, h'=0} goto loc_101;
-- for the configuration 1,0,1
loc loc_101: while hMIR223 <=  dpMIR2230  wait {dhCEBPA=-1,dhMIR223=1,dhE2F1=-1,dh=1}
 when hMIR223=dpMIR2230 do {hMIR223'=0, k'=k+1, l'=l+h, h'=0} goto loc_111;
 -- for the configuration 1,1,1
 loc loc_111: while hCEBPA >=  dnCEBPA1   wait {dhCEBPA=-1,dhMIR223=-1,dhE2F1=-1,dh=1}
 when hCEBPA=dnCEBPA1 do {hCEBPA'=0, k'=k+1, l'=l+h, h'=0} goto loc_011;
 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_000 & hCEBPA>=0 & hMIR223>=0 & hE2F1=0 & hCEBPA <=  dpCEBPA0;
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 hCEBPA,hMIR223,hE2F1,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 hCEBPA,hMIR223,hE2F1,h in hull(pln_cyc_length & r_ini) endhide;
prints "============================================================";
prints "Length of a nested cycle is:";
print hide hCEBPA,hMIR223,hE2F1,h in hull(nes_cyc_length & r_ini) endhide;
prints "============================================================";
 endif;


3rd Cycle 12122
 -- gene No0 = CEBPA

 -- gene No1 = MIR223

 -- gene No2 = E2F1
 var
 dpCEBPA0,dpMIR2230,dpE2F10,dpE2F11,dnMIR2231,dnE2F11,dnE2F12,dpCEBPA1,dnCEBPA1,dnCEBPA2: parameter;
 hCEBPA,hMIR223,hE2F1,h :analog;
 k,n,l: discrete;
 automaton auto
 synclabs: ;
 initially loc_000;
 -- for the configuration 0,0,0
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 loc_000: while hCEBPA <=  dpCEBPA0   wait {dhCEBPA=1,dhMIR223=1,dhE2F1=1,dh=1}
when hCEBPA=dpCEBPA0 do {hCEBPA'=0, k'=k+1, l'=l+h, h'=0} goto loc_100;
 -- for the configuration 0,1,0
 loc loc_010: while  hMIR223 >=  dnMIR2231  wait {dhCEBPA=1,dhMIR223=-1,dhE2F1=1,dh=1}
 when hMIR223=dnMIR2231 do {hMIR223'=0, k'=k+1, l'=l+h, h'=0} goto loc_000;
 -- for the configuration 0,1,1
 loc loc_011: while  hE2F1 >=  dnE2F11  wait {dhCEBPA=1,dhMIR223=-1,dhE2F1=-1,dh=1}
 when hE2F1=dnE2F11 do {hE2F1'=0, k'=k+1, l'=l+h, h'=0} goto loc_010;
 -- for the configuration 1,0,0
 loc loc_100: while  hE2F1 <=  dpE2F10  wait {dhCEBPA=1,dhMIR223=1,dhE2F1=1,dh=1}
 when hE2F1=dpE2F10 do {hE2F1'=0, k'=k+1, l'=l+h, h'=0} goto loc_101;
 -- for the configuration 1,0,1
 loc loc_101: while  hE2F1 <=  dpE2F11  wait {dhCEBPA=-1,dhMIR223=1,dhE2F1=1,dh=1}
 when hE2F1=dpE2F11 do {hE2F1'=0, k'=k+1, l'=l+h, h'=0} goto loc_102;
 -- for the configuration 1,0,2
 loc loc_102: while  hMIR223 <=  dpMIR2230  wait {dhCEBPA=-1,dhMIR223=1,dhE2F1=-1,dh=1}
 when hMIR223=dpMIR2230 do {hMIR223'=0, k'=k+1, l'=l+h, h'=0} goto loc_112;
 -- for the configuration 1,1,1
 loc loc_111: while hCEBPA >=  dnCEBPA1  wait {dhCEBPA=-1,dhMIR223=-1,dhE2F1=-1, dh=1}
 when hCEBPA=dnCEBPA1 do {hCEBPA'=0, k'=k+1} goto loc_011;
 -- for the configuration 1,1,2
 loc loc_112: while  hE2F1 >=  dnE2F12  wait {dhCEBPA=-1,dhMIR223=-1,dhE2F1=-1, dh=1}
 when hE2F1=dnE2F12 do {hE2F1'=0, k'=k+1} goto loc_111;
 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_000 & hCEBPA>=0 & hMIR223>=0 & hE2F1=0 & hCEBPA <=  dpCEBPA0;
 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
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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 hCEBPA,hMIR223,hE2F1,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);
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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 hCEBPA,hMIR223,hE2F1,h in hull(pln_cyc_length & r_ini) endhide;
prints "============================================================";
prints "Length of a nested cycle is:";
print hide hCEBPA,hMIR223,hE2F1,h in hull(nes_cyc_length & r_ini) endhide;
prints "============================================================";
endif;
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