Petri Net-Based Quantitative Modeling and Validation of p16-mediated Signaling Pathway

Nimet İlke Akçay

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Prof. Dr. Cem Tanova
Acting Director

I certify that this thesis satisfies the requirements as a thesis for the degree of Doctor of Philosophy in Applied Mathematics and Computer Science

Prof. Dr. Nazim Mahmudov
Chair, Department of Mathematics

We certify that we have read this thesis and that in our opinion it is fully adequate in scope and quality as a thesis for the degree of Doctor of Philosophy in Applied Mathematics and Computer Science

Assoc. Prof. Dr. Şükrü Tüzmen
Co-Supervisor

Prof. Dr. Rza Bashirov
Supervisor

Examining Committee

1. Prof. Dr. Rashad Aliyev
2. Prof. Dr. Rza Bashirov
3. Prof. Dr. Tuğrul Dayar
4. Assoc. Prof. Dr. Jens Allmer
5. Assoc. Prof. Dr. Şükrü Tüzmen

ABSTRACT

It has been a few decades since computer-based quantitative modeling methods are being applied to biological systems for enabling illustration and investigation of biological processes in a realistic way. One of the various modeling tools is Petri nets, which allow us to model pathways in biological sciences due to its extended definitions, such as hybrid functional Petri nets.

In this dissertation, by using hybrid functional Petri nets, we propose the most detailed quantitative model for p16-mediated pathway, which is a critical pathway in tumor progression. Also, simulations were conducted to validate our model, and further to make some predictions regarding the quantitative behavior of the major components and complexes in this pathway.

Keywords: hybrid functional Petri net, p16-mediated pathway, quantitative modeling, simulation

ÖZ

Bilgisayar tabanlı niceliksel modelleme metodları son birkaç on yıldır biyolojik sistemlere biyolojik süreçlerin gerçekçi bir şekilde betimlenebilmesi ve incelenebilmesi için uygulanmaktadır. Var olan birçok metodtan biri de hibrit fonksiyonel Petri ağları gibi uzantıları sayesinde biyolojik bilimlerdeki süreçsel yolları modellememizi sağlayan Petri ağlarıdır.

Bu tezde, hibrit fonksiyonel Petri ağlarını kullanarak tümör oluşumunda kritik bir biyolojik süreç olan p16 merkezli biyolojik sürecin var olan en detaylı niceliksel modelini öne sürmekteyiz. Buna ek olarak, modelimizi geçerli kılmak ve bu biyolojik sürecin temel öğelerinin niceliksel davranışları hakkında tahminlerde bulunmak amacıyla simülasyonlar yürütülmüştür.

Anahtar Kelimeler: hibrit fonksiyonlu Petri ağları, p16 merkezli biyolojik süreç, niceliksel modelleme, simülasyon

Dedicated to:

My husband, Ferhat, who has always been beside me with his endless love, in good times and in bad times.

My Fıstık, for teaching me the unconditional love and for bringing my heart so much joy, happiness and peace even on my worst days.

My parents Neriman and Erkan, and my sister Ekinnur for their endless love, support and encouragement.

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LIST OF ABBREVIATIONS

CDK	Cyclin Dependent Kinase
CKI	Cyclin Dependent Kinase Inhibitor
Cyc	Cyclin
DNA	Deoxyribonucleic Acid
E	Enzyme
ESI	Enzyme-Substrate-Inhibitor complex
G1-phase	Gap-1 phase
G2-phase	Gap-2 phase
HDN	Hybrid Dynamic Net
HFPN	Hybrid Functional Petri Net
HGP	Human Genome Project
HPN	Hybrid Petri Net
I	Inhibitor
M-phase	Mitosis phase
mRNA	messenger Ribonucleic Acid
P-invariant	Place invariant
P/T-net	Place Transition net
S	Substrate

S-phase	Synthesis phase
T-invariant	Transition invariant
UV	Ultraviolet

Chapter 1

INTRODUCTION

1.1 Motivation

Advances in biological sciences in the past few decades have revealed large number of biological data and information which need to be analyzed and interpreted. As the amount of accumulated data have increased, it has become impossible to deal with it manually. As a result, it has been essential to bring mathematical formalism and tools of computer science together in order to manage and interpret the huge amount of biological data and information. This new interdisciplinary area is referred to as 'mathematical biology' or 'biomathematics' if applied mathematical techniques are used to model biological systems; or 'bioinformatics' or 'computational biology' if tools and technologies defined in the scope of computer science are implemented in order to build structural biological databases, develop software tools, or create models and conduct simulations for being able to store, analyze, validate, and interpret biological data.

Computational modeling is a widely known method to represent a biological system in an accurate way. This is enabled by making use of already known facts and wetlab results in order to predict its dynamic behavior through observing the amounts of the components in the model via simulations. In a computational model, several variables are defined to represent the biological entities. Also, interactions between these variables are considered to reflect the dynamic behavior of the system.

Simulations are done by adjusting the variables and observing the inevident effects of changes in the system that help researchers obtain predictions about the behavior of the real system in response to different scenarios and conditions. Thus, computational modeling approach provides an advantage over wetlab experiments because of its ability to generate extensive data sets in a short time and in a low cost; computer experiments are much cheaper, faster and allow to handle huge data sets compared to experiments with cells, tissues and animals conducted in a laboratory.

In computational modeling, selection of the appropriate modeling tool is also an important decision to construct a successful model. It has been realized that a convenient modeling tool not only need to represent the biological system to obtain desired outcomes, but also allow researchers to do predictions on the behavior of the system by interpreting the results of simulations in a meaningful way. Qualitative models allow researchers to identify the structure and states of the system, while quantitative description of systems help us to fully understand the dynamic behavior of biological systems including complex interacting components.

One of the reasons in the accumulation of the available biological data over the past two decades are the advances made through the information obtained from the Human Genome Project(HGP). This was the world's largest international and collaborative biological research project [55; 95], started in 1984 and was completed in 2003. The aim of HGP was to reveal the complete mapping of all the genes of the human genome, and understand their functions. As a result of the HGP, it was announced that there are 20000-25000 genes that make up the whole human genome [21].

On the other hand, it is known that not all of the genes are at the same importance for the regulation of biological systems and survival of living organisms [12; 75]. A number of genes amongst thousands have critical importance since when mutated they tend to cause abnormalities and diseases. In cancer research, such genes are referred to as tumor suppressor genes and oncogenes. When any of these group of genes are mutated, the mutations cause loss of function or gain of function for these genes, respectively. Some specific tumor suppressor genes such as p53, p16, and PTEN and oncogenes like Ras are detected in many cancer types which increases their importance in cancer research. This dissertation study focuses on the p16 gene [89], which has a crucial role in controlling tumor suppression [77] and DNA damage; and is the major regulatory protein responsible for replicative senescence [4] and aging [50]. In addition, p16 is an important player in cell cycle regulation [92], specifically in p16-mediated signaling pathway which consists of processes regulating cell cycle during G1 phase, and plays essential role in G1/S checkpoint [10]. Disruption of p16-mediated pathway which is a key cause of human cancers [48; 79] can occur through inactivation of p16 via mutations. Because of its importance in cancer research, studies on p16 attains significance by considering p16 as a potential biomarker to detect and diagnose cancer [64; 69], which provides motivation for further research on this area.

Cell cycle is an essential biological process for proliferation of all living organisms. This process consists of a sequence of events, which are controlled by a complex network of signals and genes. In order to check and regulate the progress of the cell cycle, there exist checkpoints between neighboring phases, by which the cell division process is blocked until checkpoint requirements are met. Thus, the correct

regulation of the cell cycle is essential in order to avoid any dysfunctionality in a cell. If the occurence of any aberrancies along these sequence of events is not detected by the responsible genes in the checkpoints, then the related defect will affect downstream events negatively, which will consequently cause genetic disorders such as cancer. That is why a dozen of mathematical and computational models describing various parts of cell cycle have been constructed within the last decades [16; 19; 20; 30; 37; 66; 67; 84; 86; 87; 90]. Nevertheless, more research is needed to be performed on this area to better investigate and understand the effect of cell cycle defects on specific cancers.

1.2 Related Work

Petri nets are directed graphs that have been used to describe and analyze dynamic systems since it was first defined by Carl Adam Petri in 1962. Over the time, different extensions of Petri nets have been described for being able to illustrate and investigate stochastic, continuous or hybrid systems. As the importance and necessity of modeling biological systems increase, researchers have built Petri net models for biological systems in remarkable amount of studies [24; 25; 43; 47; 54]. Since biological systems are described by cooperation of different structured processes, it is much more suitable to implement hybrid structured Petri nets for modeling and analyzing of such systems. Modeling and simulation of a series of biological phenomena is accomplished in terms of Hybrid Functional Petri Nets(HFPN) including signaling pathway of apoptosis [54], circadian rhythms of Drosophila [54], gene regulatory mechanism of lac operon in the glycolytic pathway of E. Coli [24], validation of the p53 transcriptional activity [25], cell fate specification in the vulval development of C. Elegans [47], and the flower developmental network of Arabidopsis thaliana [43].

Modeling of the eukaryotic cell cycle by hybrid Petri net approach is proposed in [37], where both deterministic and stochastic perspectives are considered. Stochasticity is included to this model for being able to detect the change in the size of the cell and the effect of noises. This model comprises the interactions between Cdc14, Cdc20, and the complexes CycB-CDK1 and Cdh1-APC [94]. Selection of these components for the model brings out a macro-level representation of the cell cycle. However, it does not provide any observation to understand the quantitative behavior of biological entities which are involved in the regulation of the cell cycle. Moreover, in cell cycle regulation which is a very complex mechanism, hundreds of biological components play important roles and interact with each other. Thus, based on a modest size computational model, it is difficult to analyze cell cycle regulation in a quantitative manner.

1.3 Contributions

As mentioned in subsection 1.1, in this dissertation study the focus is on the p16 gene with the aim to construct and investigate the most detailed quantitative and explanatory HFPN model of p16-mediated pathway via the use of biological knowledge and latest experimental observations. Dynamic behaviors of the major proteins and protein complexes were analyzed via simulations by means of quantitative changes in response to p16 mutation and G1-dysfunction. In this sense, our model gives a remarkable insight into the key role of p16 against any aberrancy that occurs during G1 phase of the cell cycle. In addition, the regulation of replicative senescence is also taken into consideration by our presented model. After performing a series of simulations for wild type and mutated p16 against the existence and nonexistence of G1-dysfunction, the validation of our model is assured by comparing the quantitative behavior of the major components obtained from

simulation results with biological experimental data. Once validation was completed via simulation based model checking, we have used our model to conduct simulations to make predictions for unknown properties of major components of the model in terms of quantitative dynamic behaviors [3; 17].

The rest of this dissertation is organized as follows: In Chapter 2, background information about the notion of Petri Nets is presented including its basic definitions, analysis methods, and its use in modeling biological processes. In Chapter 3, the major contribution of this study, the HFPN model of p16-mediated is introduced by presenting the biological context behind this pathway, the construction of the model, and the results of our simulations. Lastly, the concluding remarks are given in Chapter 4.

Chapter 2

PETRI NETS

2.1 Background

Petri nets are a mathematical and graphical modeling tool which is applicable to many systems [60]. Concurrent, asynchronous, distributed, parallel, nondeterministic, and/or stochastic dynamic systems can be modeled and simulated by means of Petri nets. Design and analysis of Petri nets is founded on a definite and strict formalism. As a mathematical tool, Petri nets help us to represent state equations, algebraic equations, and other mathematical expressions describing dynamic systems. As being a graphical tool, Petri nets provide a visual communication aid like network diagrams. In addition, software tools developed to construct and simulate Petri net models have made Petri nets representing a powerful mechanism for modeling and analysis of particular applications. The main idea in using Petri nets is to represent states of subsystems seperately, and then to show the distributed activities of the whole system with a high effectiveness [40].

The concept of Petri nets was first introduced by Carl Adam Petri in his dissertation study on communication with automata [71], submitted in 1962 to the faculty of Mathematics and Physics at the Technical University of Darmstadt, West Germany. Since then, hundreds of articles have been published and many conferences have been organized in this field especially in Europe. Essential progress has been made in years both in theoretical research and applications of Petri nets [11; 36; 60; 80; 101].

Due to the generality and permissiveness of Petri nets, they have been proposed for a wide variety of applications. In order to learn the behavior of various problems, Petri nets have been used in a wide variety of application areas including engineering, science and industry. Performance evaluation, communication protocols, and manufacturing systems are three successful application areas. Data-flow computing systems, network control systems, logic programs, neural networks, and decision models are among the other application areas of Petri nets. In addition to these, molecular and system biology, biomedicine, and biochemistry have been added to the application areas of Petri nets especially within the last decade.

The theory of Petri nets provides a well-defined theoretical mechanism to model discrete event systems and to analyze their characteristics. Petri nets represent one of the mathematical techniques to investigate the behavior of a discrete system. The theoretical advances on Petri nets are mostly realized by the need of realistic systems in applications. Petri nets were first defined to model and analyze discrete event systems, but when the classical definition of Petri nets turned out to be too simple to add complex specifications, the definition of classical Petri net was expanded to define different types of Petri nets with extensions such as timed, colored, hierarchical, stochastic, fuzzy, continuous, hybrid, and functional. It is quite regular that Petri nets used for modeling practical problems are characterized by multiple extentions, e.g., timed and colored, or colored and hierarchical, etc.

2.2 Basic Definitions

A Petri net is a particular kind of bipartite directed graph made by three types of objects, which are places, transitions, and arcs. Places and transitions are connected each other by directed arcs. In graphical representation, places are depicted by circles

and transitions by bars or boxes. Arcs cannot directly connect nodes of the same type, i.e. arcs connect places to transitions, or transitions to places. In a Petri net, arcs are labeled with positive integers as their weights. A k-weighted arc is interpreted as a set of k parallel arcs. The weight labels for unity arcs (1-weighted arcs) are omitted.

A place is called an *input place* of a transition if there is a directed arc connecting this place to that transition. In this case, the directed arc connecting the place to the transition is called an *input arc*. Similarly, a place is an *output place* of a transition if there exists a directed arc connecting the transition to that place; and that specific arc is called an *output arc*. In applications of Petri nets, input places are used to represent the preconditions of the model, input data, input signals, resources needed, or conditions; while output places are representing postconditions of the model, output data, output signal, released resources, or conclusions; and transitions are used to model action-like events, computation steps, signal processors, tasks or jobs, logic clauses, or processors.

In Petri nets, *tokens* are used to simulate the dynamic and concurrent activities of systems. The presence of a token in a place is interpreted as holding the truth of the condition associated with that place. In order to understand the behavior of dynamic systems it is necessary to investigate a set of states and possible actions between the states. While modeling with Petri nets, system states are specified by markings. A marking can be considered as a collection of tokens distributed among the places. The *initial state* is represented by the initial marking denoted M_0. The settlement of the tokens in a Petri net can be altered by any change in the system's state, and

consequently a new marking is created. A marking is represented by an $m-$ vector M, where m is the total number of places.

Definition 1: [60] A *classical Petri net*, which is also known as *P/T-net*, is a 5-tuple $PN = (P, T, A, W, M_0)$ where

- $P = \{p_1, \dots, p_m\}$ is the finite set of places
- $T = \{t_1, \dots, t_n\}$ is the set of transitions
- $A \subseteq (P \times T) \cup (T \times P)$ is the set of arcs
- $W: A \to \mathbb{N}$ is the weight function
- $M_0: P \to \mathbb{N}$ is the intial marking

In the above definition $T \cup P \neq \emptyset$ and $T \cap P = \emptyset$.

The behaviour of a P/T-net can be described in terms of its states, where each state is represented by a marking $M \in \mathbb{N}^m$. A transition t is *enable* if each input place p has at least $w(p,t)$ tokens, where $w(p,t)$ is the weight of (p,t), the arc from p to t. Otherwise it is *disable*. When enable transition t *fires* (or *occurs*), a state $(m_1, \dots, m_n)$ is changed to the new state $(m'_1, \dots, m'_n)$ such that $m'_i = m_i \pm w(p_i, t)$. Occurrence of a transition changes distribution of the tokens, removing tokens from input places and adding tokens in output places, rather than moving tokens from input places to its output places.

A transition without any input place is called as a *source transition*, and one without any output place is called as a *sink transition*. It is possible to represent loops in Petri net models by assigning the output arc of a transition as an input arc to an upstream place of that transition. A Petri net consisting of a pair of a place and a transition is

called as a *self-loop* if the place is both an input and output place of the transition. A Petri net is called as *pure* if it has no self-loops, and a Petri net with all arc weights equal to 1 is called as *ordinary*.

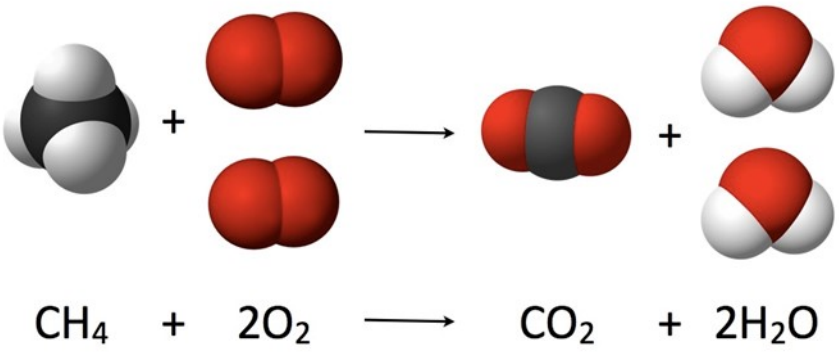

Figure 1: The combustion reaction of methane.

The combustion reaction of methane is shown in Figure 1 to illustrate Petri net terminology. This reaction uses a methane molecule CH_4 and two oxygen molecules $2O_2$ as substrates to produce a carbon-dioxide molecule CO_2 and two water molecules $2H_2O$.

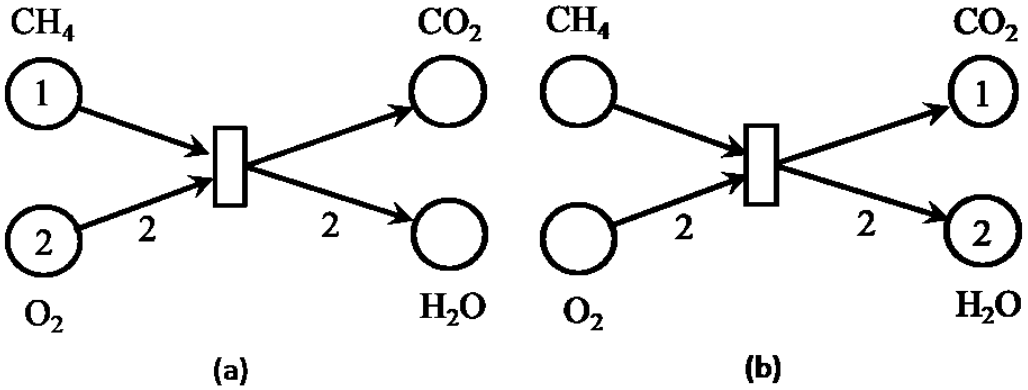

Figure 2: Petri net model of combustion reaction of methane. The two states: (a) before the reaction takes place (b) after the reaction occurs.

A Petri net model of this chemical reaction is shown in Figure 2. In this figure, circles and rectangle stand for the places and transition, respectively. A figure inside a place indicates the number of tokens in it. An inscription surrounding an arc is an arc weight indicating the number of tokens to be removed/added from/to place when the reaction takes place. This Petri net has two states, one before and another after the reaction occurs. The two states are represented by the markings $M_1 = (1,2,0,0)$ and $M_2 = (0,0,1,2)$, respectively.

When analyzing discrete event systems with classical Petri nets it is rather often that the model checking causes the state explosion, which is a fundamental problem in the study of heavily loaded discrete event systems. State explosion is an unwanted situation that leads to memory overflow. Continuous Petri nets [22] are considered as a relaxation of classical or discrete Petri nets which allow us to avoid state explosion. For similarity reason, the definition of continuous Petri nets in [22] is slightly modified and rephrased as follows.

Definition 2: A *continuous Petri net* is a 5-tuple $CPN = (P, T, A, W, M_0)$ where

- $P = \{p_1, \dots, p_m\}$ is the finite set of places
- $T = \{t_1, \dots, t_n\}$ is the set of transitions
- $A \subseteq (P \times T) \cup (T \times P)$ is the set of arcs
- $W: A \to \mathbb{R}^+$ is the weight function
- $M_0: P \to \mathbb{R}^+$ is the intial marking

In the above definition $T \cup P \neq \emptyset$ and $T \cap P = \emptyset$.

The structure of continuous Petri nets is the same to that of P/T-nets. In a continuous Petri net, the markings of places are real numbers and the firing of transitions is a continuous process. A continuous Petri net may be either autonomous with no time involved, or with firing speeds associated with transitions. The latter is suitable for performance evaluation as well as modeling dynamic biological systems.

Continuous Petri nets alone are sometimes not sufficient to model cumbersome structure of dynamic systems. For instance, biological systems comprise different structured processes. Biochemical reactions are continuous processes, while checking for presence/absence of biological phenomenon is a boolean process. On the other hand, counter-like mechanisms are represented by discrete processes. Biological processes are typical hybrid systems with coexisting different structured processes. The concept of hybrid Petri nets introduced in [46] was later developed in [23]. The formal definition of hybrid Petri nets is provided as follows by following up the style adopted in the present thesis.

Definition 3: A *hybrid Petri net* is a 6-tuple $HPN = (P, T, A, W, M_0, h)$ where

- $P = \{p_1, \dots, p_m\}$ is the finite set of places
- $T = \{t_1, \dots, t_n\}$ is the set of transitions
- $A \subseteq (P \times T) \cup (T \times P)$ is the set of arcs
- $W: A \to \mathbb{R}^+$ or $\mathbb{N}$ is the weight function
- $M_0: P \to \mathbb{R}^+$ or $\mathbb{N}$ is the initial marking
- $h: P \cup T \to \{D, C\}$ is the hybrid function indicating for every node whether it is a discrete node (sets P^D or T^D) or a continuous node (sets P^C or T^C).

In the above definition $\mathbb{N}$ and $\mathbb{R}^+$ correspond to the cases when $p_i \in P^D$ and $p_i \in P^C$, respectively. A hybrid Petri net is informally composed of two parts: the discrete part and the continuous one. The former one consists of P^D and T^D nodes, while the latter one is made of P^C and T^C nodes. Different structured fragments or components are interlinked via arcs, so that discrete place (or transition) is connected to a continuous transition (or place) and vice versa. It is also possible that one component can influence the behaviour of the other part without changing its own marking via the use of test arcs, which do not cause the removal of any content from the source place after its related transition fires.

Inhibitory arcs play important role in some applications, though it has been shown that a net fragment with inhibitory arc can be substituted by equivalent net fragment without inhibitory arc. Extended hybrid Petri nets expand the concept of inhibitory arc for the case of continuous places.

Definition 4: The definition of an *extended hybrid Petri net* [23] is somewhat similar to that of a hybrid Petri net with the exception that:

- extended Petri net might include inhibitory arcs;
- $w(p,t) \in \mathbb{R}^+ \cup \{0^+\}$ where (p,t) is inhibitory or regular arc and p is continuous place;
- $M(p) \in \mathbb{R}^+ \cup \{0^+\}$ where p is continuous place;

where 0^+ represents infinitely small positive real number.

In biological processes that can be represented by pathways, the concentration changes of components by occurence of biological processes may be depending on the amounts of some other elements in the pathway. Furthermore, the change in the

amounts of different components affecting (or affected by) a process may be different in biological pathways. HFPN were specifically proposed to model and simulate biological processes [54]. HFPN brings together all the chracteristics of P/T net, and hybrid Petri nets. In addition, it allows us to set the rates of continuous transitions as functions of concentrations in the places, which is an inherited feature from HDN [27]. Moreover, different functions can be assigned to different arcs related to a continuous transition as firing rules for the arcs, and a delay function can be assigned to a transition if needed.

The formal definition of HFPNs is given as follows.

Definition 5: [54] *HFPN* is defined based on the transition of HPN and HDN [5; 27; 28] in the following way: HFPN is composed of five types of arc; *discrete input arc, continuous input arc, test input arc, discrete output arc, and continuous output arc*. A discrete (or continuous) input arc is directed from a discrete (or continuous) place to a discrete (or continuous) transition and it consumes the content of the source place by firing. A test input arc is directed from any place to any transition and does not change the content of the source place. A discrete output arc is directed from a discrete transition to any place. A continuous output arc connects a continuous transition to a continuous place.

(1) Continuous transition: A *continuous transition* T of HFPN is composed of continuous and test input arcs $a_1, a_2, \dots, a_p$ from places $P_1, P_2, \dots, P_p$ to T and continuous output arcs $b_1, b_2, \dots, b_q$ from T to continuous places $Q_1, Q_2, \dots, Q_q$. Let $m_1(t), \dots, m_p(t)$ and $n_1(t), \dots, n_q(t)$ be the contents of $P_1, \dots, P_p$ and $Q_1, \dots, Q_q$ at time t, respectively. The rules for continuous transition T are as follows:

(a) The *firing condition* is defined as $c(m_1(t), \dots, m_p(t))$. T fires if and only if the condition remains true.

(b) For all input arcs a_i, T defines a function $f_i(m_1(t), \dots, m_p(t)) \geq 0$ determining the rate of consumption from P_i when it occurs. When a_i is a test input arc, then it is supposed that $f_i \equiv 0$ and that it does not cause change of amount to P_i. Namely, $d\frac{[a_i](t)}{dt} = f_i(m_1(t), \dots, m_p(t))$, where $[a_i](t)$ indicates the quantity removed from P_i at time t over a_i.

(c) For all output arcs b_j, T defines a function $g_j(m_1(t), \dots, m_p(t)) \geq 0$ determining the rate of addition to Q_j at time t via the output arc b_j when it fires. Namely, $d\frac{[b_j](t)}{dt} = g_j(m_1(t), \dots, m_p(t))$ where $[b_j](t)$ indicates the quantity added to Q_j at time t over b_j.

(2) Discrete transition: A *discrete transition* T of HFPN is made of discrete and test input arcs $a_1, a_2, \dots, a_p$ from places $P_1, P_2, \dots, P_p$ to T and discrete output arcs $b_1, b_2, \dots, b_q$ from T to places $Q_1, Q_2, \dots, Q_q$. Denote the contents of $P_1, \dots, P_p$ and $Q_1, \dots, Q_q$ at time t by $m_1(t), \dots, m_p(t)$ and $n_1(t), \dots, n_q(t)$, respectively. The rules for discrete transition T are as follows:

(a) The *firing condition* is determined by a predicate $c(m_1(t), \dots, m_p(t))$. T can fire if and only if the condition is true.

(b) The *delay function* is defined by function $d\left(m_1(t), \dots, m_p(t)\right)$, where $m_i(t)$ are nonnegative integer values. When firing rule holds at time t, T fires with delay $d\left(m_1(t), \dots, m_p(t)\right)$. On the other hand, when the firing rule is changed during this delay time, the transition T becomes disable and the firing condition will be reset.

(c) For all input arcs a_i, T defines a function $f_i(m_1(t), \dots, m_p(t)) \geq 0$ with nonnegative integer values $m_i(t)$, which stands for the number of tokens (integer) removed from P_i over arc a_i when T fires. It is supposed that $f_i \equiv 0$ when a_i is a test arc.

(d) For all output arcs b_j, T defines a function $g_j(m_1(t), \dots, m_p(t)) \geq 0$ with nonnegative integer values $m_i(t)$, which indicates the number of added tokens to Q_j over arc b_j.

2.3 Analysis Methods

Petri net analysis methods can be classified into three classes: (a) the state space analysis, (b) the matrix-equation approach, and (c) the reduction or decomposition techniques. Below we succinctly review each class with emphasis to possible advantages and disadvantages.

2.3.1 State Space Analysis

Given P/T-net PN, the state space analysis method is based on exhaustive search for all possible markings $M \in R(M_0)$ that are reachable from the initial marking, where $R(M_0)$ is the reachability set of the initial marking M_0. Depending on the type of the problem being considered and computational platform chosen for problem solution, the state space analysis can be implemented in the form of reachability tree, coverability tree (or coverability graph), or occurrence graph. When analysing P/T-nets with reachability tree method, one needs to draw every single marking in $R(M_0)$ to decide whether desired property holds. It is impossible to draw the reachability tree if the reachability set is infinite, expressed as $|R(M_0)| = \infty$. Unfortunately, the reachability tree method suffers from state space explosion problem, an undesired situation when the size of a state space tends to grow exponentially in the problem size, consequently leading to stack overflow and waste of active memory.

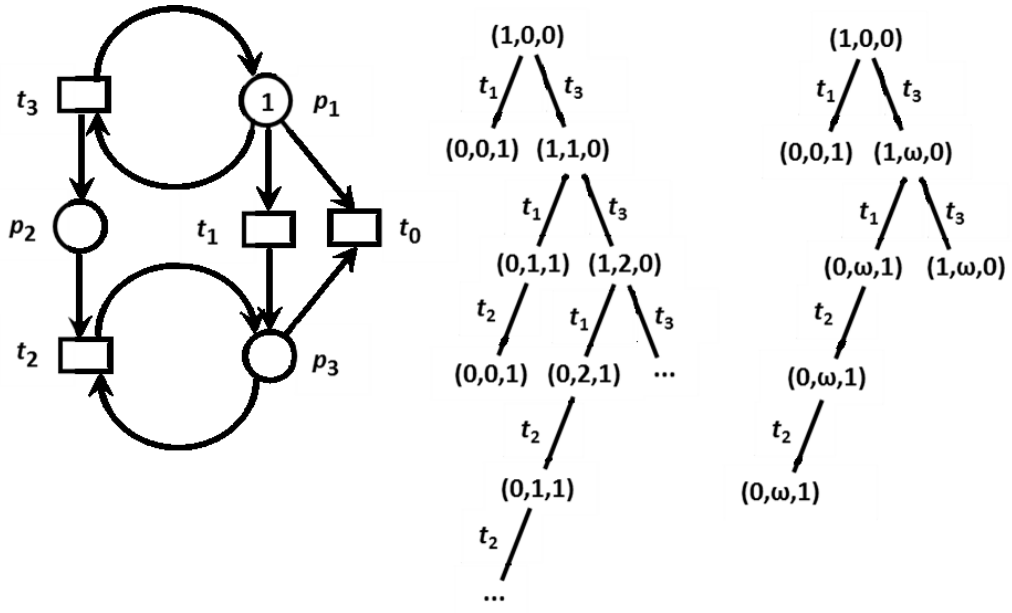

Figure 3: A P/T-net, its reachability and coverability trees.

The coverability tree method was proposed to ameliorate drawbacks of the reachability tree method. The coverability tree method has certain advantages over the reachability tree method. It is rather easy to store the coverability tree since it is always finite while reachability tree can grow infinitely. The coverability tree method has the same analysis power as the reachability tree method. However, the coverability tree suffers from the same state space explosion problem. As an example, a P/T-net, its reachability tree, and coverability tree are illustrated in Figure 3.

It is sometimes more convenient to draw state space starting with some specified marking, which can be in general different than the initial marking. The resulting state space is called occurrence tree or graph. CPN Tools [42] and other software tools support drawing occurrence graph for P/T-nets as well as for Petri nets with color, time and hierarchy extensions.

The state space analysis should be applied to all classes of nets, but application is limited to modest-size nets due to the complexity and the state-space explosion.

2.3.2 The Matrix-Equation Approach

The matrix-equation approach is perhaps the most powerful among existing P/T-net analysis methods. Unlike the state space method, which remains mainly as enumeration of all reachable or coverable markings, the matrix-equation approach is applied to subset of a reachability set $R(M_0)$. The matrix equation approach is based on linear algebra, in which the Petri net structure is represented as a matrix, called incidence matrix. The incidence matrix I is a matrix $I: P \times T \rightarrow \mathbb{Z}$, indexed by P and T such that $I(t,p) = w(p,t) - w(t,p)$. A *place vector* x is defined as $x: P \rightarrow \mathbb{Z}$ and indexed by T. Likewise, a *transition vector* y is defined as $y: T \rightarrow \mathbb{Z}$ and indexed by P. A place vector is called *P-invariant* if it is a nontrivial solution of the system of linear equations $x \cdot I = 0$. Similarly, a transition vector is called *T-invariant* if it is a nontrivial solution of the system of linear equations $I^T \cdot y = 0$.

The P-invariants has essential practical application in biological systems [35]. It was reported that the P-invariant is a token conserving fragment of the net. Expressed in another way, a P-invariant is a collection of places over which the weighted sum of tokens is constant and independent of any firing. In terms of metabolic networks, P-invariants reflect substrate conservations, while in signal transduction networks P-invariants often correspond to the several states of a given biological component such as protein or protein complex. A place belonging to a P-invariant is obviously bounded, since it is token preserving.

The entries of a T-invariant specify a multiset of transitions whose firing reproduce a specified marking. Given a T-invariant, partially ordered sequence of the transitions may contribute to a deeper understanding of the Petri net behavior in sense that the entries in a T-invariant may also be interpreted as the relative firing speeds of permanently and concurrently occurring transitions. It was discussed that [35] this activity level corresponds to the steady state behavior.

P-invariants and T-invariants are often used to check for reachability of a destination marking M_d from the initial marking M_0. It has been shown [60] that the existence of a nonnegative integer solution x (or y) satisfying the matrix equation $x \cdot I = \Delta M$ (or $I^T \cdot y = \Delta M$) where $\Delta M = M_d - M_0$ is a necessary but, in general, not sufficient condition for M_d to be reachable from M_0. For acyclic P/T-nets the above condition is also sufficient.

2.3.3 The Reduction or Decomposition Techniques

The reduction or decomposition techniques are used to convert the original Petri net into more compact one by preserving all behavioral properties of the original Petri net. The decomposition technique is useful when it is hard to verify the property with the given Petri net. For instance, it is extremely hard to apply matrix-equation approach to high level Petri nets. There is however a way to check reachability property by matrix-equation approach in high level Petri net. To do so, the given high level Petri net needs to be decomposed into its equivalent P/T-net, and then matrix-equation approach should be used to check for reachability.

2.4 Modeling Biological Processes with Petri Nets

The history of Petri nets implemented for modeling of biological processes goes back to the mid of 90s when Reddy in his pioneering paper proposed a method to

represent metabolic pathways [78]. Since then there is an increasing interest in this research field, at least as far as it can be told from the number of published papers [8; 18; 24; 25; 35; 37; 43; 47; 54]. Quantitative modeling was particularly on focus of researchers who aimed on deeper understanding of biological interactions within metabolic pathways, signal transduction pathways and gene regulatory networks. Below we describe fundamentals of quantitative modeling of biochemical reactions for which Petri Nets are implemented. [1; 14; 35]

2.4.1 Modeling Unimolecular, Bimolecular and Reversible Reactions

Unimolecular reaction or *first-order* reaction, which is schematically represented as $A \rightarrow B$, describes the conversion of an unstable molecule A into stable one B. The radioactive decay is an example to unimolecular reaction. Petri net in Figure 4 characterizes a unimolecular reaction.

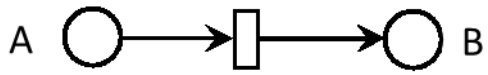

Figure 4: Petri net model of a unimolecular reaction.

Bimolecular reaction or *second-order* reaction describes interaction between two substrates A and B to form the product C. Corresponding Petri net is represented in Figure 5.

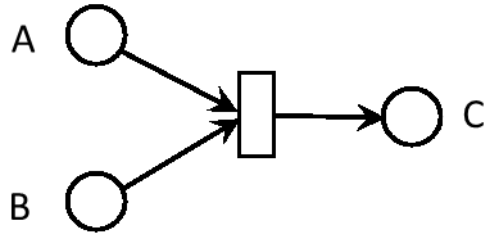

Figure 5: Petri net model of a bimolecular reaction.

In principle any reaction is reversible. Under pressure of thermodynamic parameters a bimolecular reaction may progress in one direction, converting substrates into product, or in another direction, converting product into substrates. A *reversible* or "for and back" reaction $A + B \leftrightarrow C$, which is also called *equilibrium* reaction, is represented by the Petri net illustrated in Figure 6.

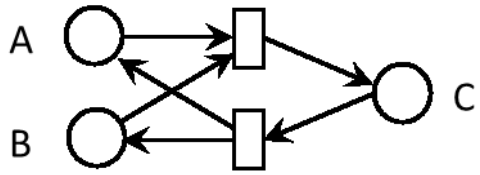

Figure 6: Petri net model of a reversible reaction.

Starting with the initial concentrations of substrates A, B and product C the reaction makes progress until reaching an equilibrium point or *steady-state* at which the time derivative of concentration is zero. A biological system can be at steady-state or be driven away from it by any small perturbation. Steady states play important role in stability analysis of biological systems.

2.4.2 Modeling Enzymatic Reactions

In biochemistry, an *enzyme* also known as a catalyst is a small molecule that catalizes chemical reactions. The concentration of an enzyme remains the same before and after the reaction. This peculiarity of enzymes puts additional distinctive feature to enzyme-catalized reactions in sense these reactions do not demonstrate allosteric effect such as cooperativity. The enzyme-catalized reaction can be represented by $E + S \leftrightarrow ES \rightarrow P + E$, where E stands for enzyme, S for substrate, ES for intermediate enzyme-substrate complex, and P for product. For corresponding Petri net see Figure 7.

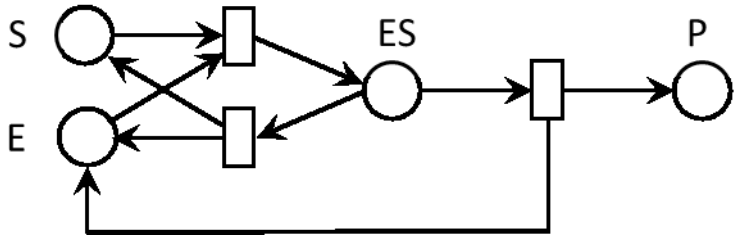

Figure 7: Petri net representation of an enzymatic reaction.

2.4.3 Modeling Activation of Proteins via Phosphorylation

In biological processes, some proteins need to be activated by other proteins in order to perform their tasks. In biological signaling pathways, kinases are considered to be proteins having phosphate adding (phosphorylating) abilities in order to activate (or sometimes deactivate) other proteins. In order to model such processes, the Petri net in Figure 7 is slightly modified to add a dephosphorylation transition, since it is known in biology that phosphorylation is a reversible process [31]. The corresponding Petri net representation is shown in Figure 8.

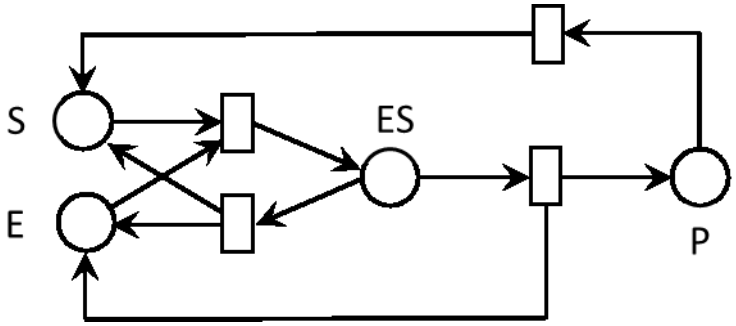

Figure 8: Petri net representation of activation via phosphorylation.

2.4.4 Modeling of Enzyme Kinetics with Inhibitors

In many biosystems it is often required to suppress specific enzymes by proteins called inhibitors in order to knock out selected reactions. Depending on the way the reaction proceeds, we usually distinguish between competitive, uncompetitive, and noncompetitive inhibition mechanisms [1]. In the following, the three types of

inhibition mechanisms are briefly explained, and corresponding Petri nets are presented.

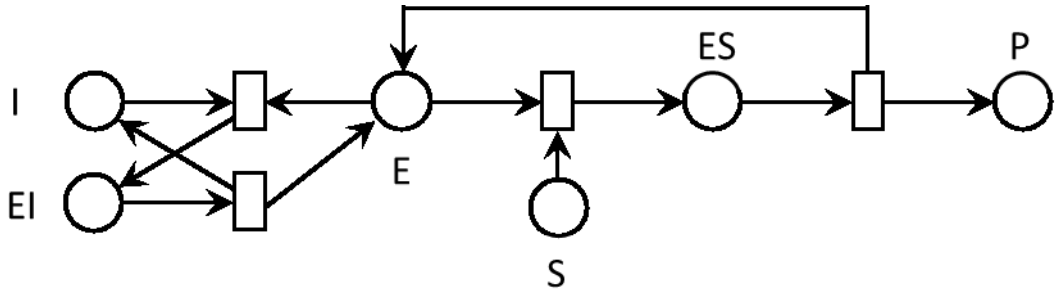

Figure 9: Petri net representation of an enzymatic reaction with competitive inhibition.

In the case of a competitive inhibition, the substrate S and inhibitor I are structurally very similar and compete for the same enzyme E, forming either enzyme-substrate complex ES or enzyme-inhibitor complex EI, but not more higher order complex ESI. Corresponding Petri net is illustrated in Figure 9. As a biological example for a competitive inhibition, we can consider p16-CycD-CDK4/6 pathway in which p16 competes with Cyclin D for binding to CDK4/6 complex in the case when a dysfuntion occurs [41; 83]. In modeling of this mechanism, p16 is considered to be the inhibitor, Cyclin D to be the substrate, and CDK4/6 complex to be the enzyme.

In uncompetitive inhibition, the inhibitor binds only to the enzyme-substrate complex, that is, inhibitor and substrate do not compete for enzyme. As a result a catalytically inactive enzyme-substrate-inhibitor complex arises, but no enzyme–inhibitor complex is formed. Petri net model for the case of uncompetitive inhibition is depicted in Figure 10. p21-CycD-CDK4/6 pathway can be given as an example of an uncompetitive inhibition. The inhibition by p21 occurs via its binding to CycD-

CDK4/6 complex which means there is no competition between the inhibitor p21 and the substrate Cyclin D for binding to the enzyme complex CDK4/6 [6; 29; 33; 82].

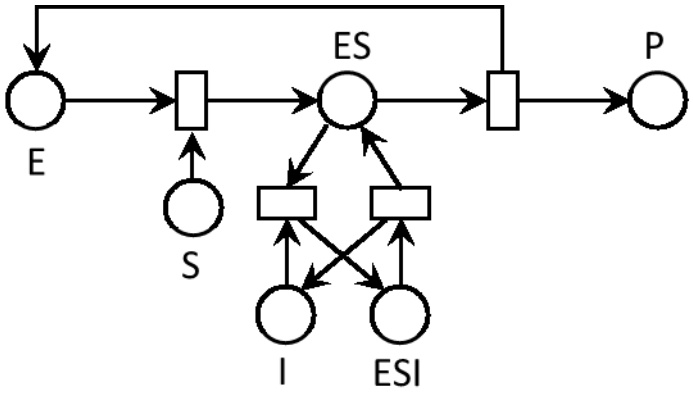

Figure 10: Petri net representation of an enzymatic reaction with uncompetitive inhibition.

In noncompetitive inhibition, the enzyme-inhibitor, enzyme-substrate and also enzyme-substrate-inhibitor complexes are formed, which is illustrated in Figure 11. For this type of inhibition mechanism, the p14-MDM2-p53 pathway can be considered as an example, where p14 is placed as the inhibitor, MDM2 as the enzyme, and p53 as the substrate in the model. It is known from the biological literature that the complexes p14-MDM2, MDM2-p53, and p14-MDM2-p53 can be formed in this pathway: p14-MDM2 is formed for stabilizing functional p53 by inhibiting its ubiquitination [38; 45; 73]; MDM2 binds to p53 to inactivate the transcription function of p53 and to promote p53 degradation by ubiquitination [44; 70; 98]; and p14-MDM2-p53 trimer complex can also be formed to assure the transcriptional activity of p53 on genes MDM2 and Bax [25].

Thus, when modeling this specific pathway the formed complexes p14-MDM2, MDM2-p53, and p14-MDM2-p53 can be assumed to be enzyme-inhibitor, enzyme-substrate, and enzyme-substrate-inhibitor complexes respectively.

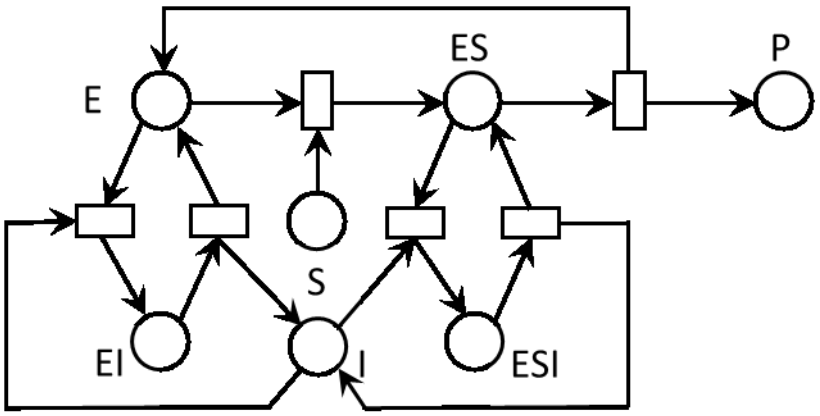

Figure 11: Petri net representation of an enzymatic reaction with noncompetitive inhibition.

Chapter 3

QUANTITATIVE MODELING OF P16-MEDIATED PATHWAY WITH HYBRID FUNCTIONAL PETRI NETS

In this chapter, HFPNs are implemented to model and validate p16-mediated signaling pathway which has its role in the G1 phase of the cell cycle.

3.1 Biological Context

A gene is a basic unit of heredity that is located in a specific segment of DNA, a double-stranded molecule that twists around its axis to form a helical structure. A gene itself is not a functional unit but it contains instructions for making proteins, which are large molecules that play essential role in survival of living organisms. Protein production, also known as gene expression, is a two-step process. Transcription is the first step of protein production, in which the instructions in the gene are copied from DNA to mRNA. In the second step, which is known as translation, a protein is created from mRNA.

Proteins rarely act alone, and in order to be active they need to interact with other proteins by either forming molecular complexes via binding or being phosphorylated by kinases. The protein complexes resulting from binding processes usually exist in the form of a dimer (or binary complex), a trimer (or ternary complex) and a tetramer (or quaternary complex).

Proteins are continuously synthesized and degraded in a living organism. Protein degradation, also known as natural degradation, is a way to discard and recycle unnecessary proteins, and thus to keep a protein concentration at a predefined level. Ubiquitination or ubiquitin-mediated protein degradation is a mechanism that degrades or destroys the abundance of major proteins that no longer serve its purpose in an organism [100]. Natural degradation and ubiquitination proceed at different reaction rates. Gene expression on the other hand requires a reliable mechanism to turn genes on and off, and consequently regulate mRNA levels. This is achieved by regulating the balance between transcription and mRNA degradation [26].

3.1.1 Cell Cycle

A cell is perhaps the smallest functional unit that exhibits all the characteristics of life. Human cells, with the exception of mature red blood cells, have nuclei surrounded by cytoplasm. Nuclei contain the genetic material of the cell, which is known as DNA. Biological components in a cell are moved between nucleus and cytoplasm. Transporting biological components from nucleus into cytoplasm and vice versa is referred to as nuclear export and nuclear import, respectively [53].

The cell cycle is an ordered sequence of events that leads to cell division, which is a fundamental biological process essential for the proliferation of all living organisms. The cell cycle events are classified into discrete periods or phases. These phases are aligned respectively in the order of G1 (gap period 1); S (synthesis); G2 (gap period 2); and M (mitosis) as can be seen in Figure 12. During G1 phase, based on information received from extracellular environment, cells decide whether to proliferate or not. It is in this phase cells start growing to reach twice their size. Cyclins, CDKs, and CKIs are key proteins that control cell cycle. DNA integrity is

always under attack of environmental factors such as UV radiation and tabacco smoke. Damaged DNA is a potential source for mutations and can lead to unregulated cell proliferation, a key cause of cancer. Intact or repaired DNA permits DNA replication which occurs in the S phase. G2 phase separates end of DNA synthesis from initiation of mitosis. Cyclins, CDKs, and CKIs act in this phase to prepare cells for mitosis. Finally, M phase results in the production of two identical daughter cells from a single parent cell.

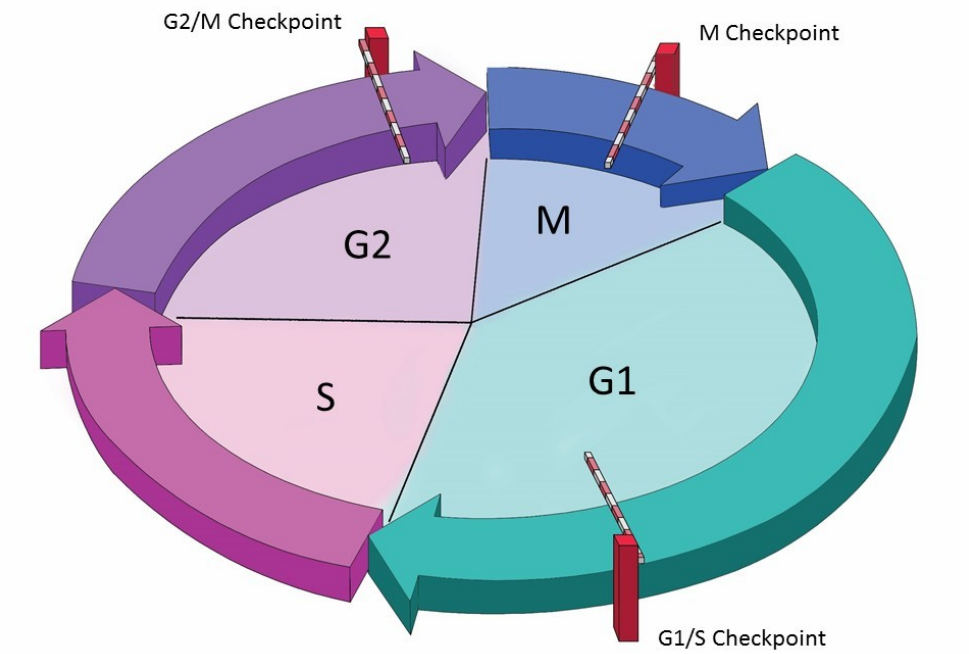

Figure 12: Schematic representation of the human cell cycle with its phases and checkpoints

3.1.2 Cyclins and CDKs

Advances in understanding the cell cycle in the last two decades are tightly related to the discovery of Cyclin Dependent Kinases(CDK) and cyclins.

As a family of protein kinases, CDKs were discovered as the cell cycle regulators. CDKs bind to their specific regulatory proteins called cyclins. CDKs have little kinase activity without their cyclin partners. Only the cyclin-CDK complexes are

active kinases. CDKs phosphorylate their substrates on serines and threonines, so they are serine-threonine kinases [59]. CDK levels remain fairly constant throughout the cell cycle and most regulation is achieved post-translationally. The four major mechanisms of CDK regulation involve cyclin binding, CAK phosphorylation, regulatory inhibitory phosphorylation, and binding of CDK inhibitory subunits (CKIs) [58].

Protein complexes are very important in biological processes because they help reveal the structure-functionality relationship in complex interactions within biological systems [51]. Cyclins are substrate proteins which bind and thus activate CDKs. As four classes of Cyclins; A, B, D, and E type cyclins have been observed in human cells, each centered around one Cyclin-CDK complex. The CycD-CDK4/6 complex is responsible for progression in G1 phase, CycE-CDK2 complex regulates passage through G1/S transition, CycA-CDK2 complex promotes the progression in S phase, and CycB-CDK1 complex activity drives the G2/M transition.

3.1.3 CKIs

Although Cyclin-CDK complexes play a critical role in cell cycle regulation, another class of proteins exist to control these cell cycle regulators. In human cells these are called CDK Inhibitors or CKIs, for short. Under certain circumstances CKIs bind to and inhibit the corresponding CDKs activity. Damaged DNA, cell cycle abnormality and environmental stresses are among circumstances that force CKIs to inhibit CDKs activity.

CKIs are classified into two major families, INK4 and Cip/Kip [62]. The INK4 family is named so for ability of its proteins to inhibit CDK4-6 complex from binding to cyclin D. Four such proteins are p15, p16, p18 and p19. In contrast to

INK4 proteins, Cip/Kip family proteins are more broadly acting inhibitors, whose actions affect the activities of cyclin D-, E-, and A-dependent kinases. The Cip/Kip family includes p21, p27 and p57. All of aforesaid inhibitors play fundamental role in tumor suppression, and they are among the tumor suppressor genes. Inactivation of CKIs' tumor suppressing functions by gene mutations is one of the most frequent alterations found in human cancers.

3.1.4 Cell Cycle Checkpoints

Failures in the DNA replication and environmental stresses such as UV radiation and tobacco smoke might cause DNA damage. Damaged DNA can result in loss of genetic information and mutations, destroying the control of cell proliferation. Cells use complex signaling pathways called the cell cycle checkpoints to control the accuracy and consistency of cell division, detect and maintain DNA damage, and alleviate stresses on genomes [99]. The checkpoints halt progression into the next phase of the cell cycle until damaged DNA has been precisely repaired. The most studied cell cycle checkpoints are G1/S and G2/M checkpoints.

3.1.5 Replicative Senescence

Human cells are not immortal as they undergo a finite number of cumulative population doublings, then enter a state termed as replicative senescence. It was observed that normal human cells permanently can divide 50±10 times (Hayflick limit) before they succumb to replicative senescence [34]. In human cells, replicative senescence is a powerful tumor suppressive mechanism, which also contributes to ageing.

However, if the tumor supression mechanism does not work properly due to some mutations on tumor suppressor genes, the Hayflick limit is bypassed and cells evade

replicative senescence. For a cell with extended lifespan, susceptibility to malignant progression greatly increases, which leads us to consider replicative senescence as a barrier to tumor formation.

3.1.6 G1/S Checkpoint: The p16-Rb and p21-Rb Signaling Pathways

G1/S checkpoint of the cell cycle consists of two main signaling pathways which are p16-mediated and p21-mediated pathways.

The tumor suppressor genes p16 and p21 play key roles in detection and repair of DNA damage and keeping track of replicative senescence. p16 and p21 utilize their functions in G1 phase and G1/S checkpoint, respectively. Figure 13 is a schematic illustration of p16- and p21-mediated control mechanism occurring in human cells. In wild-type human cells, CDK4 binds to CDK6 to form CDK4/6 dimer, which in turn is activated by binding cyclin D and constructing CycD-CDK4/6 trimer. This active CDK-Cyclin complex hypo-phosphorylates Rb-E2F-DP complex to lead cyclin E being expressed, which in turn forms a complex with CDK2. The complex CDK2-CycE hyper-phosphorylates Rb-E2F-DP complex [63]. This hyper-phosphorylation causes the transcription factor E2F being released from Rb-E2F-DP complex and thus allowing cells to enter S phase by initiating the transcription of the genes having role in the S phase of the cell cycle.

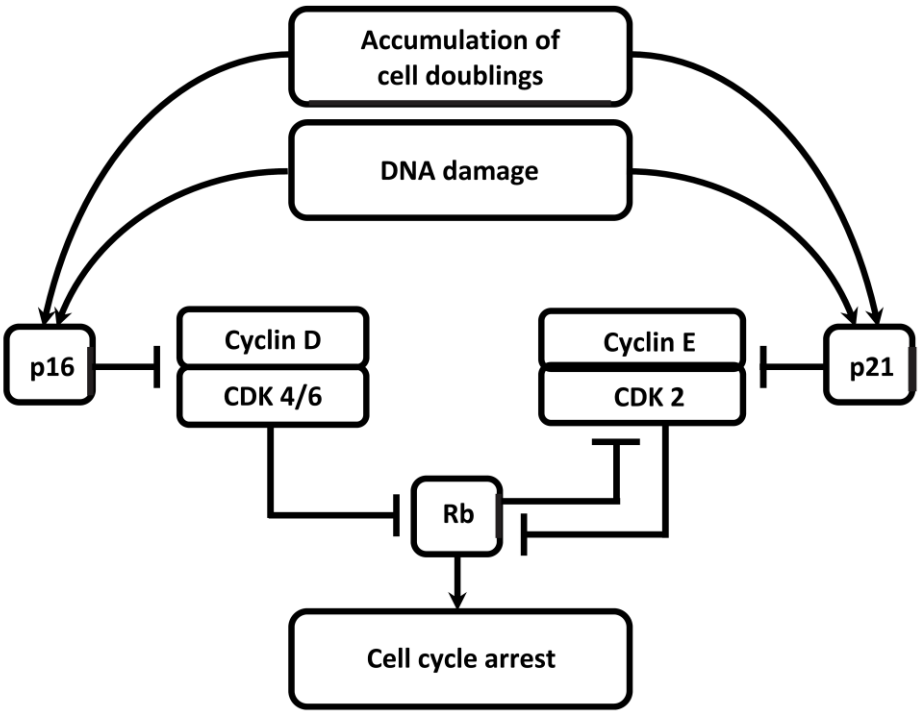

Figure 13: Schematic illustration of p16- and p21-mediated control mechanism regulating DNA damage and replicative senescence.

When number of accumulated cell doublings reaches the Hayflick limit [34], p16 receives a signal on replicative senescence. As a result p16 competes with Cyclin D [81] in order to bind CDK4/6 and thereby to inhibit binding of CDK4/6 to Cyclin D to prevent Rb phosphorylation [53; 97]. This leads to irreversible arrest in G1 phase of cell cycle.

When DNA damage is detected, the action of p16 again targets CDK4/6 and results in arrest in G1 phase until DNA damage is repaired. Inactivation of tumor suppressor gene p16 occurs through its mutation. Mutated p16 gene loses its gatekeeper role at G1 phase which might cause uncontrolled cell division leading to cancer [7]. When p16 is mutated in a case in which it should act as an inhibitor, it cannot prevent the hypo-phosphorylation of Rb-E2F-DP complex, but in the next step functional p21 takes responsibility by preventing the hyper-phosphorylation of

Rb-E2F-DP complex, and thus E2F cannot be released to initiate S phase. If both p16 and p21 are inactivated by mutations, then the blocking mechanism cannot work properly in the case when DNA damage occurs, which will cause the damage being carried to other phases of the cell cycle and eventually the formation of a tumor.

3.2 Model Construction

HFPN model of p16-Rb pathway is created by the use of biological information in the literature [2; 7; 9; 26; 34; 49; 52; 53; 61; 68; 88; 93; 96; 97; 99; 100] which is briefly discussed in section 3.1. In this model, it is assumed that the four major proteins of p16-Rb pathway; cyclin D, p16, CDK4 and CDK6 are synthesised in accordance with the central dogma of molecular biology: mRNA transcribed from DNA is then translated into protein. The abundance of mRNA that no longer used for protein production is destroyed by mRNA degradation. All unnecessary proteins and protein complexes are also discarded by protein degradation. In the Petri net model, transcriptions are considered as source transitions, while degradations are represented by sink transitions. In Figure 14, this biological phenomena is illustrated by means of Petri nets for CDK4. Similar net fragments are constructed for also p16, CDK6 and Cyclin D. In addition, Cyclin D is subject to proteasome-mediated degradation which is also known as ubiquitination.

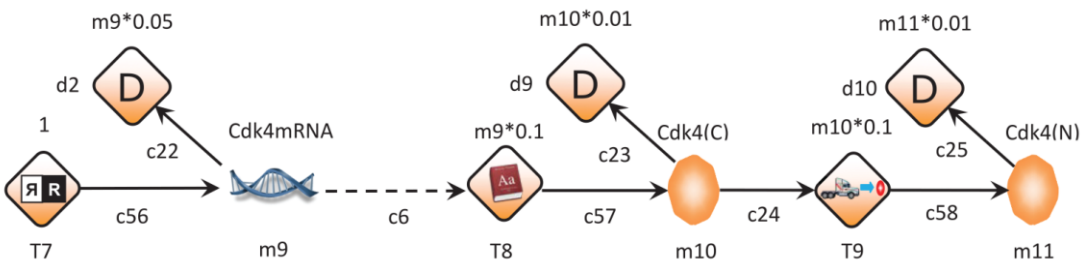

Figure 14: Central dogma of biology illustrated for CDK4.

		G1-DYSFUNCTION	
		YES	NO
P16 MUTATION	YES	❖ Mutated p16 loses its inhibitory function. ❖ If the reason of dysfunction is replicative senescence, cells evade replicative senescence, gaining immortality, or an extended replicative lifespan, which leads to tumor progression in an organism. ❖ If the reason of dysfunction is DNA damage, there is no way to arrest cellcycle at G1 phase and maintain damaged DNA. Damaged DNA results in loss of genetic information and mutations.	❖ Mutated p16 loses its inhibitory function. ❖ CycD binds to CDK4/6 resulting in phosphorylation of Rb, causing successive cell division until Hayflick limit is reached or DNA damage arises. ❖ When the Hayflick limit is reached, cells evade replicative senescence, gaining immortality, or an extended replicative life span which leads to tumor progression in an organism. ❖ When DNA is damaged there is no way toarrest cell cycle at G1 phase and maintain damaged DNA. Damaged DNA results in loss of genetic information and mutations.
	NO	❖ Wild-type p16 inhibits binding of CDK4/6 with CycD by forming a complex p16CDK4/6, and thereby preventing Rb phosphorylation. ❖ If the reason of dysfunction is replicative senescence, cells enter in to a state of irreversible growth arrest. ❖ If the reason of dysfunction is DNAdamage, cell cycle is arrested at G1 phase until damaged DNA is maintained.	❖ CycD binds to CDK4/6 resulting in phosphorylation of Rb, causing successive cell division until Hayflick limit is reached in a healthy cell cycle state.

Figure 15: Classification of biological events with respect to p16 mutation and G1-dysfunction.

Our model is centered upon the gatekeeper role of p16 in regulating p16-Rb pathway. For this reason, we construct our model by considering scenarios that may happen in the case when p16 is mutated or nonmutated. Also, we consider other abnormalities that may occur within this pathway such as mutations of genes other than p16, DNA damage, or environmental stress by naming all of these as G1-dysfunction. Cascade of biological events induced by each of four possible scenarios regarding p16 mutation and G1-dysfunction are described in Figure 15.

Based on the facts given in Figure 15, the inhibition mechanism controlled by active p16 is modeled by the use of a competitive inhibition skeleton explained in section 2.4.4 since p16 and Cyclin D compete for binding to CDK4/6 in this mechanism.

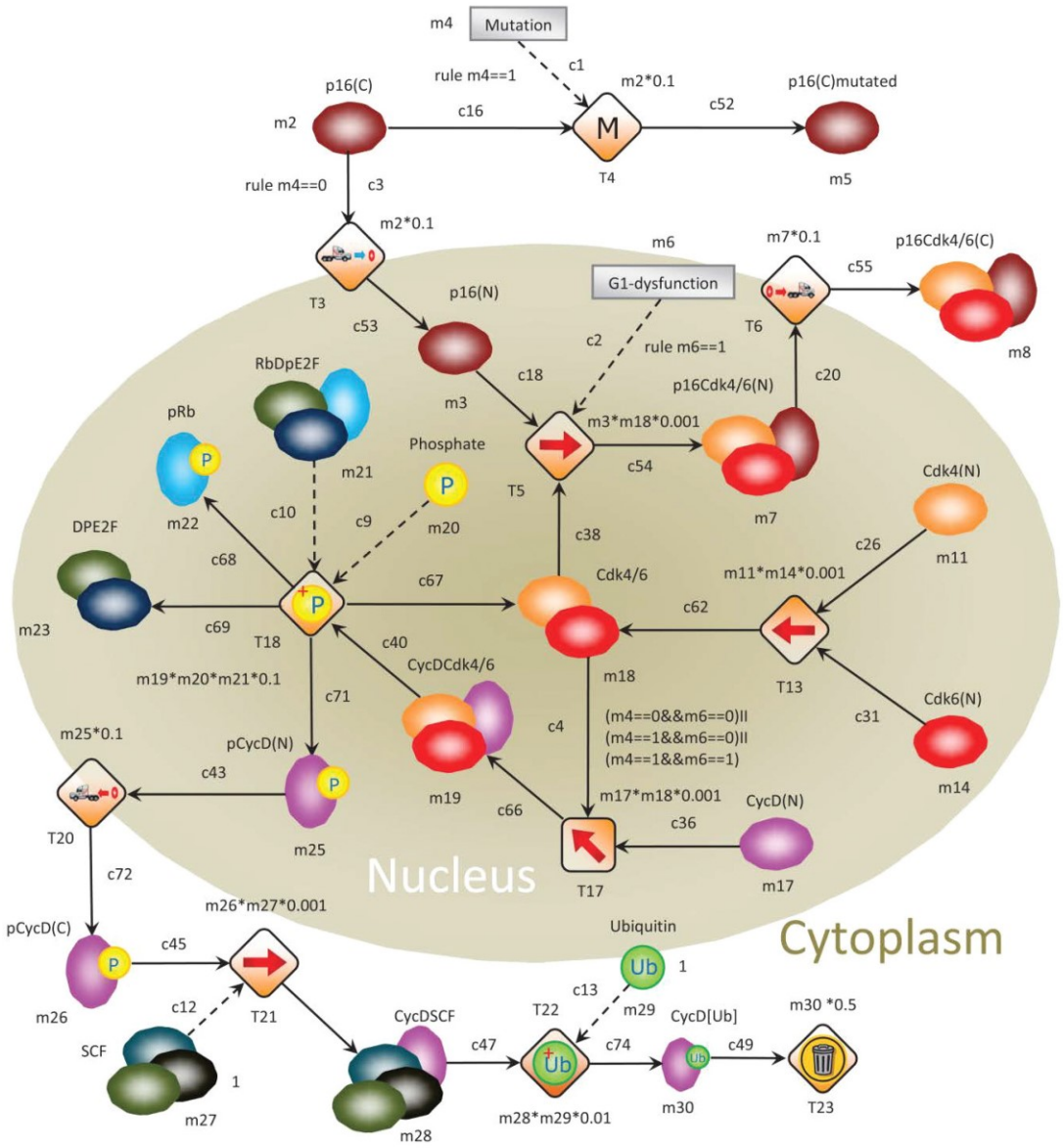

Figure 16: HFPN model of p16-mediated pathway in human cell cycle.

The skeleton HFPN model of the p16-mediated pathway is given in Figure 16. Graphical description of protein syntheses and other satellite data discussed so far are discarded in this model for the sake of clarity, and for being able to emphasize the main important processes of this pathway and molecular interactions between the major proteins.

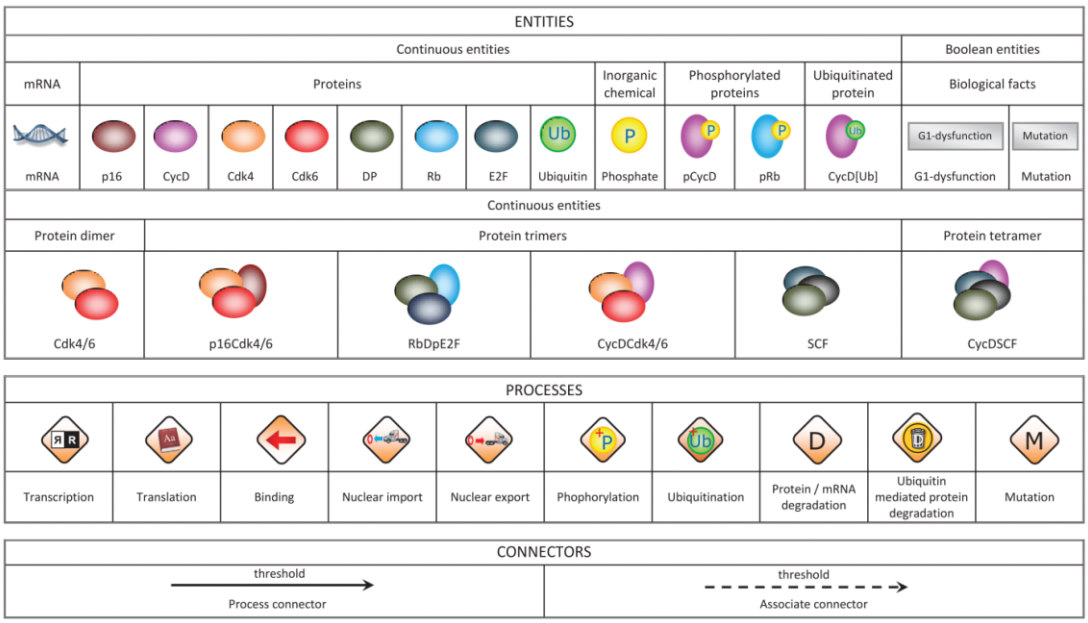

Figure 17: The elements used in HFPN model for p16-mediated pathway.

HFPN model of p16-Rb pathway is composed of 28 continuous entities representing mRNAs, proteins, protein complexes, ubiquitin, phosphate, ubiquitinated proteins and phosphorylated proteins; 2 generic entities indicating presence/absence of p16 mutation and G1-dysfunction; 44 continuous processes standing for transcription, translation, nuclear transport, binding, phosphorylation, ubiquitination, mRNA degradation, natural degradation and mutation; 74 process and associate connectors. The model comprises 30 variables labelled $m1$ to $m30$, two of which are introduced to indicate presence/absence status of mutation of p16 ($m4$) and G1-dysfunction ($m6$), and remaining 28 variables are defined to measure the concentrations of biological components. The types and identifiers used in the present model are specified in Figure 17 with the only notice that we introduce similar labels for nucleic and cytoplasmic concentrations of a biological component, e.g. nucleic and cytoplasmic concentrations of cyclin D are respectively assigned inscriptions CycD(N) and CycD(C). Relationship between entities and biological components is illustrated in Table 1.

Table 1: Correspondence between biological components and HFPN entities

Entity Name	**Entity Type**	**Variable**	**Initial Value**	**Value Type**
p16mRNA	Continuous	$m1$	0	Double
p16(C)	Continuous	$m2$	0	Double
p16(N)	Continuous	$m3$	0	Double
Mutation	Generic	$m4$	true/false	Boolean
p16mutated	Continuous	$m5$	0	Double
G1dysfunction	Generic	$m6$	true/false	Boolean
p16CDK4/6(N)	Continuous	$m7$	0	Double
p16CDK4/6(C)	Continuous	$m8$	0	Double
CDK4mRNA	Continuous	$m9$	0	Double
CDK4(C)	Continuous	$m10$	0	Double
CDK4(N)	Continuous	$m11$	0	Double
CDK6mRNA	Continuous	$m12$	0	Double
CDK6(C)	Continuous	$m13$	0	Double
CDK6(N)	Continuous	$m14$	0	Double
CycDmRNA	Continuous	$m15$	0	Double
CycD(C)	Continuous	$m16$	0	Double
CycD(N)	Continuous	$m17$	0	Double
CDK4CDK6	Continuous	$m18$	0	Double
CycDCDK4/6	Continuous	$m19$	0	Double
Phosphate	Continuous	$m20$	1	Double
RbDpE2F	Continuous	$m21$	1	Double
pRB	Continuous	$m22$	0	Double
DpE2F	Continuous	$m23$	0	Double
SPhaseGenes	Continuous	$m24$	0	Double

pCycD(N)	Continuous	$m25$	0	Double
pCycD(C)	Continuous	$m26$	0	Double
SCF	Continuous	$m27$	1	Double
CycDSCF	Continuous	$m28$	0	Double
Ubiquitin	Continuous	$m29$	1	Double
CycD[Ub]	Continuous	$m30$	0	Double

Biological processes depend on many parameters including type of substrates, type of culture, environmental factors, etc. It is hard, if not impossible, to determine exact rates based on data coming from biological laboratory experiments. It is known that mostly two identical experiments lead to different observations [57]. The results of wetlab experiments regarding rate measurements may sometimes be contradictory. In this work, the rates of biological phenomena are estimated according to their relative rates. We firstly preset rate of transcription to 1, and then set the rates of remaining biological phenomena by comparing them with the rate of transcription. The correspondence between processes and biological phenomena; and process rates adopted in the present work, that are comparable to those in the literature [24; 25], are presented in Table 2 and Table 3.

Table 2: Correspondence between biological phenomena and HFPN processes

Biological Phenomenon	**Process**	**Process Type**	**Process Rate**
Transcription of p16mRNA	$T1$	Continuous	1
Translation of p16	$T2$	Continuous	$m1 \times 0.1$
Nuclear import of p16	$T3$	Continuous	$m2 \times 0.1$
Mutation of p16	$T4$	Continuous	$m2 \times 0.1$
Binding of p16(N) and CDK4/6	$T5$	Continuous	$m3 \times m18 \times 0.001$
Nuclear export of p16CDK4/6	$T6$	Continuous	$m7 \times 0.1$
Transcription of CDK4mRNA	$T7$	Continuous	1
Translation of CDK4	$T8$	Continuous	$m9 \times 0.1$
Nuclear import of CDK4	$T9$	Continuous	$m10 \times 0.1$
Transcription of CDK6mRNA	$T10$	Continuous	1
Translation of CDK6	$T11$	Continuous	$m12 \times 0.1$
Nuclear import of CDK6	$T12$	Continuous	$m13 \times 0.1$
Binding of CDK4 and CDK6	$T13$	Continuous	$m11 \times m14 \times 0.001$
Transcription of CycDmRNA	$T14$	Continuous	1
Translation of CycD	$T15$	Continuous	$m15 \times 0.1$
Nuclear import of CycD	$T16$	Continuous	$m16 \times 0.1$
Binding of CDK4/6 and CycD	$T17$	Continuous	$m17 \times m18 \times 0.001$
Phosphorylation of RB	$T18$	Continuous	$m19 \times m20 \times m21 \times 0.1$
Transcription of S phase genes	$T19$	Continuous	$m23 \times 1$
Nuclear export of pCycD	$T20$	Continuous	$m25 \times 0.1$
Binding of pCycD and SCF	$T21$	Continuous	$m26 \times m27 \times 0.001$
Ubiquitination of CyclinD	$T22$	Continuous	$m28 * m29 * 0.01$
Degradation of CycD[Ub]	$T23$	Continuous	$m30 \times 0.5$

Table 3: Natural degradations in the HFPN model

Biological Phenomenon	**Process**	**Process Type**	**Process rate**
Degradation of mRNAs	$d1 - d4$	Continuous	$mi \times 0.05$
Degradation of proteins	$d5 - d21$	Continuous	$mi \times 0.01$

To keep the concentration of related mRNAs at specified level associate connectors are used between mRNA entries and related transcription processes. Information on connectors including firing styles, firing scripts, and connector types are described in Table 4.

Table 4: Connectors in the HFPN model

Connector	**Firing Style**	**Firing Script**	**Connector Type**
$c1$	Rule	$m4 == 1$	input association
$c2$	Rule	$m6 == 1$	input association
$c3$	Rule	$m4 == 0$	input process
$c4$	Rule	$(m4 == 0 \;\&\&\; m6 == 0) \;\|\|$ $(m4 == 1 \;\&\&\; m6 == 0) \;\|\|$ $(m4 == 1 \;\&\&\; m6 == 1)$	input process
$c5 - c13$	Threshold	0	input association
$c14 - c49$	Threshold	0	input process
$c50 - c74$	Threshold	0	output process

The model allows rule-based processing of biological events in accordance with previously mentioned four scenarios in Figure 15. Note that $T4$ and $m4$ control the status of mutation. Likewise, $m6$ checks the presence of dysfunction in G1 phase. When p16 is mutated, the rule $m4 == 1$ enables $T4$. Occurrence of $T4$ arrests p16 in cytoplasm, indicating that p16 is no longer functional as an inhibitor. Otherwise, $T3$

occurs in accordance with rule $m4 == 0$, transporting p16 from cytoplasm to nucleus. When dysfunction occurs in Gl phase, in appliance with rule $m6 == 1$, p16 inhibits formation of CycD-CDK4/6 complex. Complete set of rules for Rb phosphorylation is represented as inscription on $c4$ in Table 4.

HFPN model is constructed in Cell Illustrator 5.0, a professional version that is licensed to Eastern Mediterranean University. A screen snapshot of HFPN model is illustrated in Figure 18.

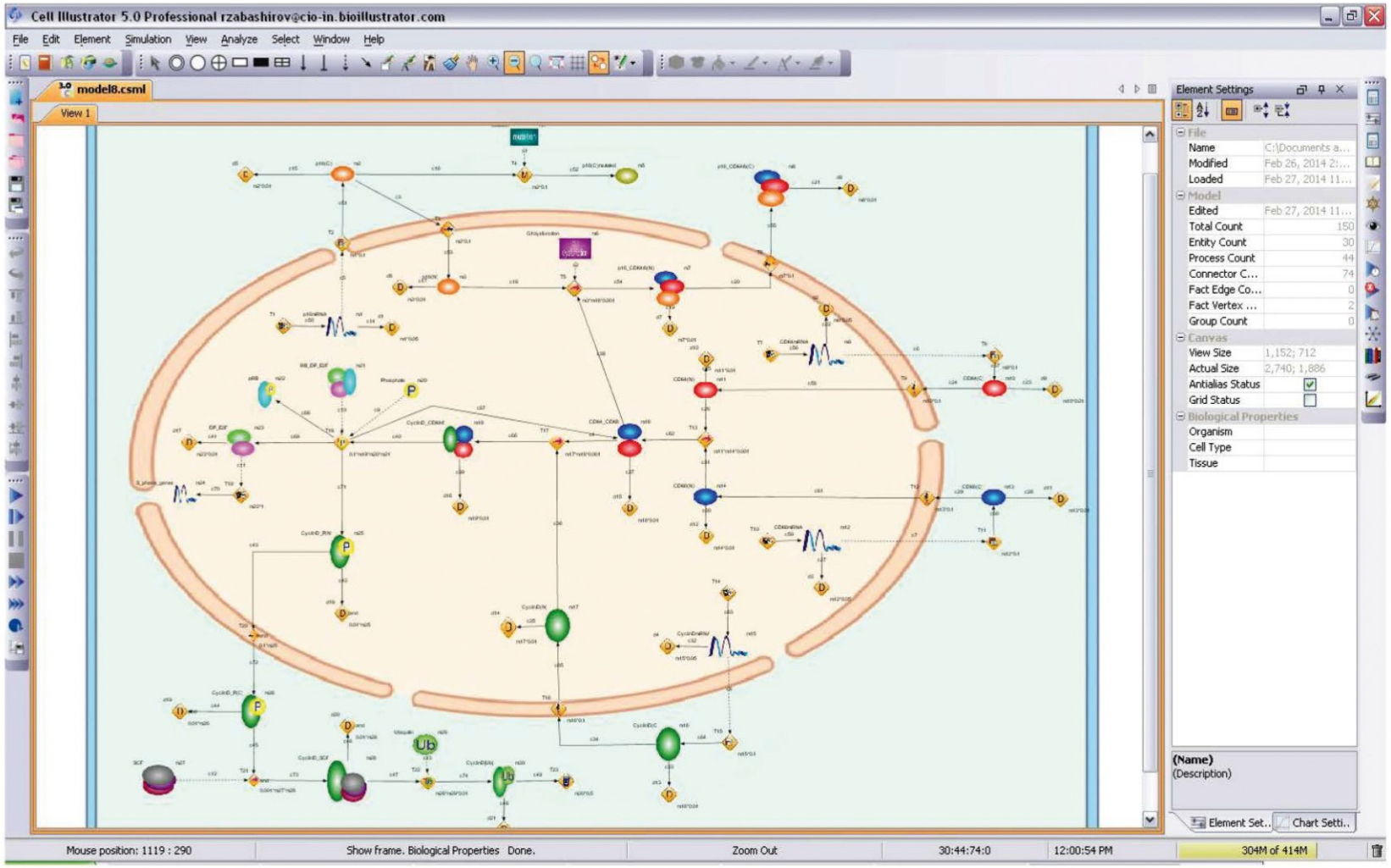

Figure 18: Cell Illustrator screen snapshot illustrating p16-mediated signaling pathway

3.3 Simulations and Validation

By the use of the HFPN model described in the previous section, the concentrations of the major biological components of the p16-Rb pathway are estimated by conducting simulations in accordance with the following four scenarios:

(a) p16 is active and G1-dysfunction does not occur

(b) p16 is active and G1-dysfunction occurs

(c) p16 is inactivated and G1-dysfunction does not occur

(d) p16 is inactivated and G1-dysfunction occurs.

The plots of concentrations are created against time units called Petri time or pt, for short. For being able to compare the simulation results for all components, simulations are performed at the same pt sampling interval and consequently the same simulation granularity. We continued simulating until 500 pt for clarity of observations, although asymptotic behaviors of measured concentrations were observed within 200 pt.

3.3.1 Simulation Results and Validation

3.3.1.1 Results for the gene p16

The simulation results obtained for the levels of mutated p16 in cytoplasm, p16 in nucleus, and p16CDK4/6 complex in cytoplasm and nucleus are given in Figure 19. The first row in this figure includes the concentrations of these components in wild-type case in which there is no p16 mutation and dysfunction (Figure 19-a). Since there is no reason for p16 to arrest the cell cycle, the components p16(C)mutated, p16CDK4/6(C) and p16CDK4/6(N) have no concentration at all (Figure 19-I, III, IV-a). The levels of p16 in nucleus increases and reaches to a steady-state concentration level (Figure 19-II-a). The second scenario for these components is for the existence

of a dysfunction when p16 is nonmutated in which p16 binds to CDK4/6 and is transported to cytoplasm with this complex to prevent the formation of CycD-CDK4/6 complex and thus to arrest the cell cycle by stopping Rb phosphorylation. As expected, our simulation results show no concentration of mutated p16 (Figure 19-I-b), and some steady-state amount of p16 in nucleus, and p16CDK4/6 complex in cytoplasm and nucleus (Figure 19-II,III,IV-b). The obtained concentration level of p16 in nucleus for this case is lower than the wild-type case since some amount of p16 binds to CDK4/6. By comparing the concentration levels of p16CDK4/6 complex in cytoplasm and nucleus (Figure 19-III-b and Figure 19-IV-b respectively), we are able to conclude that p16CDK4/6 is accumulated in cytoplasm.

In tumor progression, inactivation of p16 by the mutations has been reported as a critical event. Loss of p16 function is observed in so many human cancers [48]. Although very few studies analyzed cellular localization of p16, there is evidence that remarkable amount of p16 concentration in cytoplasm is monitored in some neoplasms [32; 65]. Since study of cytoplasmic localization and accumulation of p16 is a recent event, the mechanisms behind the arrest of p16 in cytoplasm have not been clarified yet, nevertheless there exist a few hypotheses explaining the cytoplasmic accumulation of p16. In the case when p16 is inactivated by mutations, simulation results are plotted by monotonic stable steady-state of p16 cytoplasmic concentration with approximately linear rate of growth (Figure 19-I-c,d). The concentration of mutated p16 which is localized in cytoplasm reaches its peak level at 750 close to the end of simulation time. Mutations on p16 usually arise in the form of promoter hypermethylation [39], homozygotic deletion [74; 76] or loss of heterozygosity [91]. Here in this work the types of mutations are not considered

since the impact of mutation types to concentration behavior of p16 needs to be further investigated by biological experiments. In the light of the consequences given in Figure 15 which are triggered by the loss of p16 function, it is known that p16 is arrested in cytoplasm and its transportation to nucleus is prevented when it is inactivated by mutations. As explained in section 3.1.6, the inhibitory function of p16 occurs by its binding to CDK4/6 in nucleus to prevent CycD-CDK4/6 binding and thus Rb phosphorylation and passage to the S phase. Being localized in cytoplasm, p16 cannot act as an inhibitor in the case when a dysfunction occurs within the cell since its inhibitory mechanism does not work due to its mutation. Even if Hayflick limit is reached or DNA is damaged, p16 is not able to bind CDK4/6 and the formation of CycD-CDK4/6 complex and phosphorylation of Rb cannot be stopped. Therefore, p16 cannot accomplish the necessary cell cycle arrest when it is inactivated by mutations and there is a dysfunction in the cell. Our simulation results in Figure 19-c,d provide a good fit to the scenarios in which p16 is mutated by showing no accumulation of p16 in nucleus (Figure 19-II-c,d), and consequently no accumulation of p16CDK4/6 in nucleus (Figure 19-IV-c,d) and in cytoplasm (Figure 19-III-c,d). As expected, the simulation results in Figure 19-c,d do not show any difference no matter if there is G1-dysfunction or not which is a consistent result with the related scenarios in Figure 15.

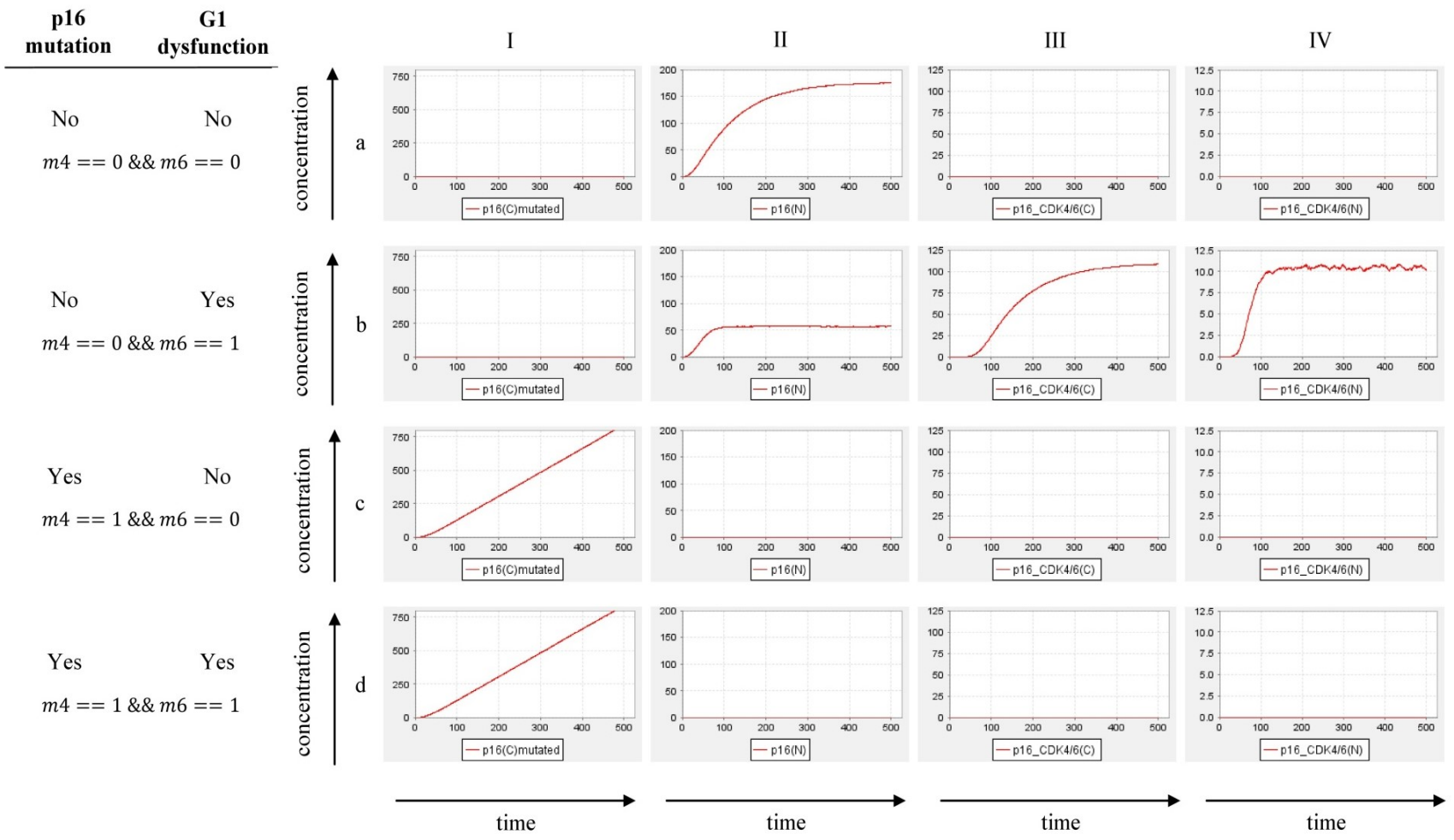

Figure 19: Simulation results for p16(C)mutated, p16(N), p16CDK4/6(C) and p16CDK4/6(N).

3.3.1.2 Cyclin D Related Results

In the literature, there does not exist an absolute consensus among researchers regarding Cyclin D levels before, during, and after the proliferation and different observations about Cyclin D levels are reported. Some researchers claim that Cyclin D is subsequently expressed throughout cell cycle and its levels are more constant unlike the other cyclins A, B, and E. [13; 15; 72] . On the other hand, it is also reported that Cyclin D is completely disrupted by proteosome-mediated degradation at the end of G1 phase [56]. However, the majority of the researchers suggest that Cyclin D levels in wild type cells are high during G1 phase in response to growth factors to initiate DNA synthesis, but then to allow for efficient DNA synthesis it is supressed to low levels during S phase and finally it is induced again in G2 phase to support proliferation [85; 102].

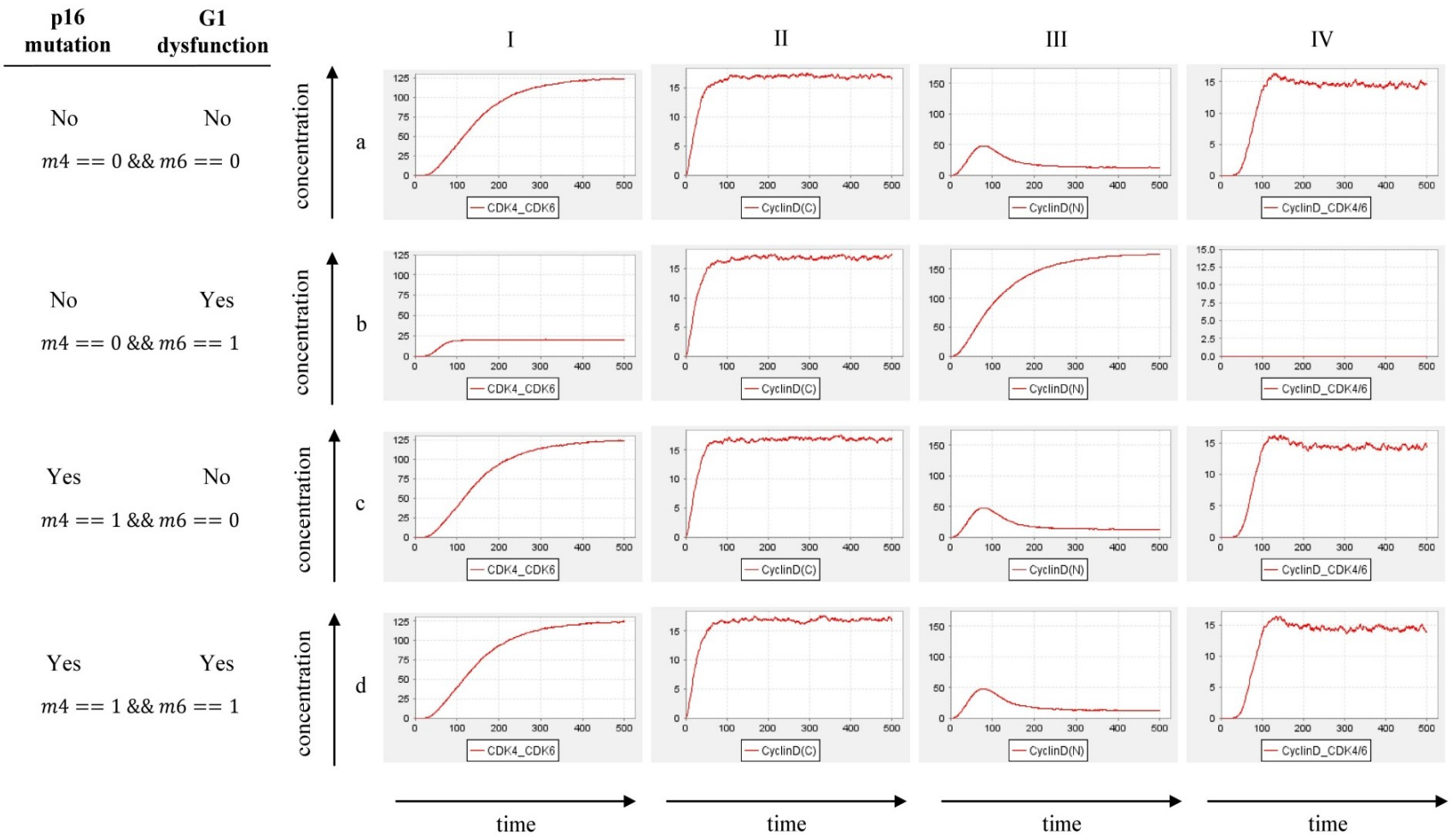

Figure 20: Simulation results for CDK4/6, CycD(C), CycD(N) and CycDCDK4/6.

Our simulation results for the concentration behavior of Cyclin D in nucleus based on the four scenarios is given in Figure 20-III. We observe the asymptotic behavior of Cyclin D in nucleus when there is no mutation on p16 and G1-dysfunction takes place. In this situation, Cyclin D enters a steady constant state close to the concentration units of 175 (Figure 20-III-b). The reason of this behavior is that in order to arrest cell cycle, functional p16 inhibits binding of Cyclin D to CDK4/6 by binding to CDK4/6 itself to form p16CDK4/6 complex. Thus, p16 prevents Rb phosphorylation and consequently the ubiquitination of Cyclin D and this results in accumulation of high levels of Cyclin D concentration in nucleus. Furthermore, when we compare the concentration levels of p16CDK4/6 complex in nucleus (Figure 19-IV-b) with its cytoplasmic concentration (Figure 19-III-b) it is observed that this complex is mainly accumulated in cytoplasm rather than in nucleus. This is rather an interesting result since to the best of our knowledge, it has not been reported in the literature so far.

In wild-type case in which there is no mutation on p16 and G1-dysfunction does not occur, Cyclin D successfully binds to CDK4/6 which causes Rb being phosphorylated and Cyclin D is degraded by proteosome-mediated ubiquitination. The concentration of Cyclin D in nucleus is induced rapidly so that it reaches a peak level at the level 50 in approximately 75 pt. Then a rapid decrease is observed due to proteosome-mediated ubiquitination of Cyclin D as shown in Figure 20-III-a. Thus, our simulation results are in agreement with the observations obtained in [85; 102]. It is neither subsequently expressed to keep the concentration at a constant level as it is suggested in several papers [13; 15; 72], nor completely disrupted as it is reported by other researchers [56]. It is also observed that in this scenario p16 is accumulated in

nucleus (Figure 19-II-a). By comparing two cases in Figure 19-II-a and Figure 20-III-b, it can be observed that maximum levels of Cyclin D and p16 concentrations in the nucleus are the same, which is close to the level of 175 units.

In the remaining two scenarios in which p16 is mutated, the concentration behavior of Cyclin D (Figure 20-III-c,d) is similar to that of the wild-type case. This is because even if G1-dysfunction occurs inactivated p16 is unable to arrest the cell cycle and Cyclin D levels decrease due to proteosome-mediated ubiquitination.

3.3.1.3 Results on CDKs

It is broadly known that, once produced, a CDK is present thrughout the cell cycle since it is an enzyme. The fact that CDK levels remain relatively constant throughout the cell cycle was also reported in [52; 58]. Our simulation results in Figure 21 is in agreement with the literature by reflecting very little variation for the CDK protein levels.

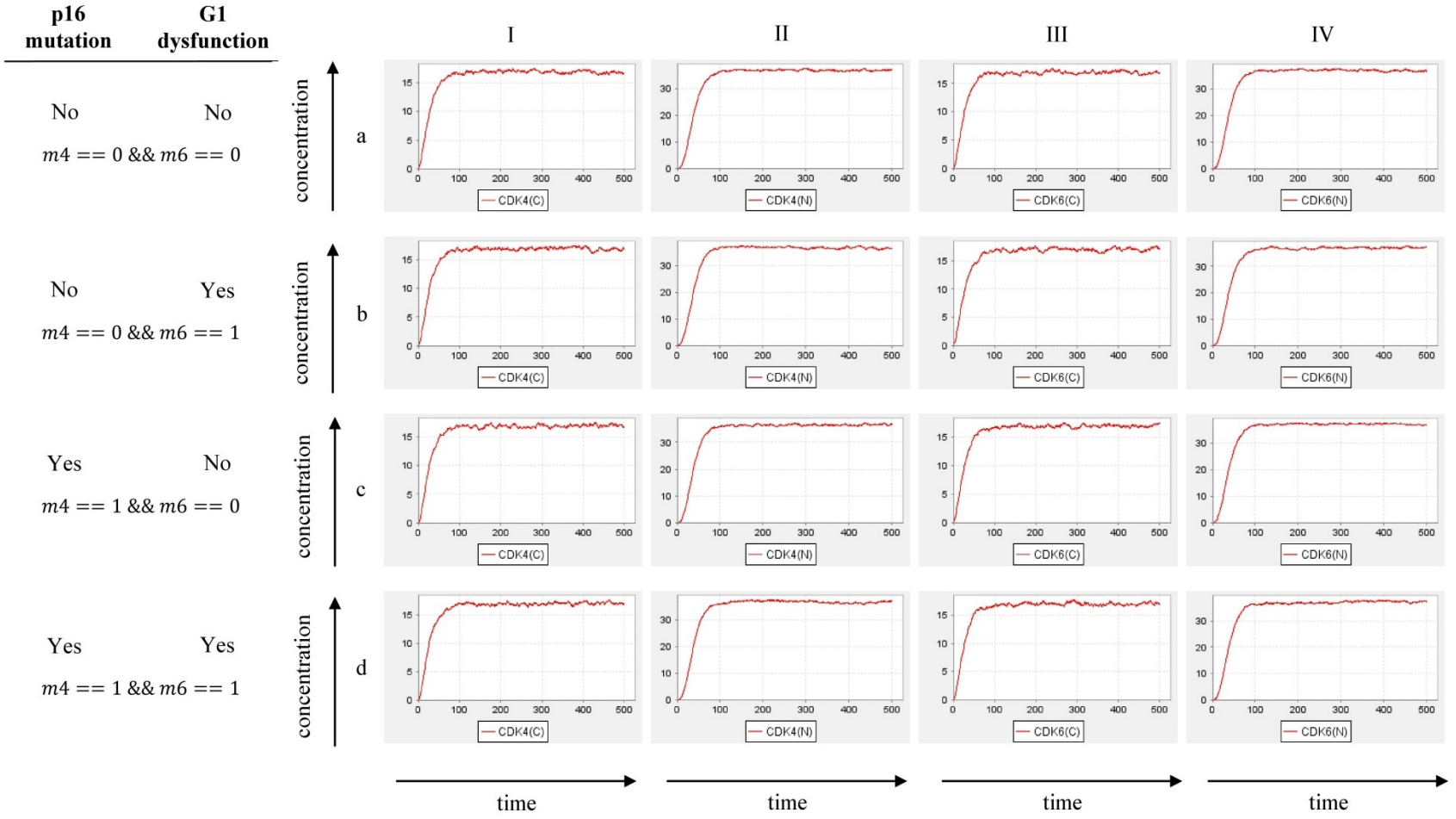

Figure 21: Simulation results for CDK4(C), CDK4(N), CDK6(C) and CDK6(N).

Simulation results for the dimer CDK4/6 is given in Figure 20-I. By considering the fact that equal amounts of cyclin D (Figure 20-III-b) and p16 (Figure 19-II-a) concentrations are available for binding with CDK4/6 coupled with a constant rate of binding reaction, it could be expected to result in equal amount of CDK4/6 concentrations left after forming resulting complexes. However, we obtained a surprising result in Figure 20-I: the amount of remaining CDK4/6 concentration is as high as 125 in cases (a), (c) and (d), and it is as low as 20 in case (b). A reasonable explanation for this observation could be the following: When DNA damage is present p16 binds to CDK4/6 to prevent Rb phosphorylation and to arrest cell cycle until damaged DNA is maintained, or when replicative senescence takes place p16 arrests cell cycle continuously. Thus, dynamic behavior of CDK4/6 for case Figure 20-I-b supports this idea by reflecting low levels of CDK4/6 concentration remained after forming p16CDK4/6.

Chapter 4

CONCLUSION

In this dissertation, HFPNs have been exploited to construct the computational model of the p16-mediated pathway, which is an important biological pathway playing key roles in human cell cycle, senescence, and aging. By incorporating HFPN and biological signaling pathways, we provided additional benefits to both fields: The number of succesfully implemented HFPN applications were broadened, and on the biological side a quantitative-based wider understanding about the regulation of the human cell cycle were attained.

Through the extended properties of HFPNs, we were able to represent the dynamic interactions between the components by proposing the most detailed quantitative computational model so far for this pathway. Simulations were conducted for the major components in this pathway under four possible scenarios with respect to inactivation of the p16 gene by mutations, DNA damage, and replicative senescence. Obtained simulation results are in accordance with the available findings from biological experiments in the literature. Moreover, we have interpreted our simulation results in a meaningful way in the cases where there were no existing experimental observations to compare with. Via such interpretations, we were able to propose predictions about the quantitative behaviors of the major components in this pathway. Our main findings include:

(1) p16 inactivation by mutations cause an increase on its cytoplasmic concentration, regardless of another dysfunctionality of the major components in this pathway (Figure18-I-(c,d)).

(2) For wild-type cells, simulation results for Cyclin D reveal that during G1 phase Cyclin D levels are high for initiation of DNA synthesis, but during S phase its concentration is diminished to low levels (Figure 20-III-a).

(3) In the case where p16 is active and a dysfunctionality of the major components in this pathway occurs in G1 phase, a large amount of the p16CDK4/6 complex is accumulated in cytoplasm rather than in the nucleus (Figure 19-III-b, Figure 19-IV-b).

(4) For wild type cells, we obtained simulation results showing that active p16 is located in the nucleus with high levels of accumulation (Figure 19-II-a).

(5) When p16 is functional and DNA damage or replicative senescence occurs, then Cyclin D is mainly accumulated in the nucleus (Figure 20-III-b).

(6) Based on our simulation results on CDK4 and CDK6, it was concluded that concentrations of CDK proteins fluctuate with very little amounts during the cell cycle (Figure 21).

(7) Concentration levels of the dimer CDK4/6 is high in all scenarios (Figure 20-I-(a,c,d)) except for the case when p16 is active and DNA is damaged or replicative senescence occurs (Figure 20-I-b).

As a future study, we plan to focus on some specific cancer types for constructing their detailed quantitative computational HFPN models.

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