Data-Driven Modelling of SUMOylation and deSUMOylation in Response to Cellular Stresses

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Declaration

I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and Acknowledgements.

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Abstract

An overarching question in biomedical systems that of understanding how cell fate is determined when exposed to various environmental and metabolic stresses such as heat shock, hypoxia, oxidative and genotoxic stresses. In recent years, experimental works have shown that SUMOylation, one of the protein post-translational modifications (PTMs), plays a key and essential role in most cellular response to extreme stresses. It follows that a better understanding of the cellular stress responses pathways and the associated SUMOylation regulatory machinery is imperative, which would, for example, lead to new developments for the treatment of diseases associated with environmental and metabolic stresses. SUMOylation is also a highly dynamic and reversible process, in which the modified proteins can be deconjugated from SUMOylated target proteins by SUMO/Sentrin-Specific Proteases (SENPs), termed as deSUMOylation. To probe the role of SUMOylation and deSUMOylation processes in cellular response to extreme stresses, this thesis aims to develop dynamic models of SUMOylation-dependent synergism of cellular stresses response, focusing on two stresses: heat shock and hypoxia stresses.

The new mathematical models of SUMOylation and deSUMOylation process in response to heat shock and hypoxia cellular stresses have been developed. It is the first time to propose the mechanism of SUMOylation in combination with different cellular stresses response. The system of ordinary differential equation models developed in this thesis is based on the underlying SUMOylation signalling network inferred from experimental findings and mass action kinetics. Data-driven methods are adopted to optimise the model parameters, including the genetic and differential evolution algorithms. Global sensitivity analysis, a variance-based Sobol's approach, is used to estimate parameters, which is the study of how the uncertainty in the output of a mathematical model can be divided and allocated to different sources of uncertainty in its inputs. Furthermore, experiments have been conducted to validate the developed model to generate time-course data on the cell

response to heat shock and hypoxia stresses. The results show that the predicted results agree closely with the experimental findings.

Abbreviations

AD Alzheimer's Disease

APP Amyloid precursor protein

Aβ Amyloid-β

ARNT Aryl Hydrocarbon Receptor Nuclear Translocator

DBD DNA Binding Domain

DE Differential Evolution Algorithm

DESI1 deSUMOylating Isopeptidase 1

DMEM Dulbecco's Modified Eagle's Medium

DP Denatured Protein

DNA Deoxyribonucleic Acid

E1 SUMO Activating Enzyme

E2 SUMO Conjugating Enzyme

E3 SUMO Ligation Enzyme

EPA Extreme Pathway Analysis

ER Endoplasmic Reticulum

FIH Factor Inhibiting of HIF

FBS Fetal Bovine Serum

GA Genetic Algorithm

GSA Global Sensitivity Analysis

HCC Hepatocellular Carcinoma

HD Huntington's Disease

HIF Hypoxia-inducible Factor

HIFs Hypoxia-inducible Factors

HIF1α Hypoxia-inducible Factor 1α

HIF1β Hypoxia-inducible Factor 1β

HS Heat Shock

HSF Heat Shock Factor

HSFs Heat Shock Factors

HSF1 Heat Shock Factor 1

HSP Heat Shock Protein

HSPs Heat Shock Proteins

HSP40 Heat Shock Protein 40

HSP70 Heat Shock Protein 70

HSP90 Heat Shock Protein 90

HSE Heat Shock Element

HSR Heat Shock Response

HRE Hypoxia Response Element

HR Homologous Recombination

KD Kennedy Disease

MIMs Molecular Interaction Maps

MFPs Misfolded Proteins

MC Monte Carlo

NBD N-terminal ATP Binding Domain

NLS Nuclear Localisation Signal

NEM N-Ethylmaleimide

ODEs Ordinary Differential Equations

PTM Post-translational Modification

PHD Proline Hydroxylase Domain

PHDs Prolyl Hydroxylases Domains

PD Parkinson's Disease

PN Protein Homeostasis Network

PML Promyelocytic Leukaemia

RNA Ribonucleic Acid

ROS Reactive Oxygen Species

RNS Reactive Nitrogen Species

SBD Substrate-binding Domain

SENP SUMO-Specific Protease

SENPs SUMO-Specific Proteases

SDS Sodium Dodecyl Sulphate

SIMs SUMO-interacting Motifs

sHSP small Heat Shock Protein

SUMO Small Ubiquitin Modification

SUMOs Small Ubiquitin-like MOdifiers

TD Trimerisation Domain

TLB Triton X-100 Lysis Buffer

Ulps Ubiquitin-like Proteases

USPL1 Ubiquitin-specific Protease-like 1

VHL von Hippel-Lindau

List of Publications and Presentations

1. M. Zhang, A. Zhao, C. Guo, and L. Guo, "A combined modelling and experimental study of heat shock factor SUMOylation in response to heat shock," *Journal of theoretical biology*, vol. 530, p. 110877, 2021.

2. M. Zhang, Y. Zhang, A. Zhao, C. Guo, and L. Guo, "Towards data-driven modelling of sumoylation following heat shock," in *2020 7th International Conference on Biomedical and Bioinformatics Engineering*, pp. 16–21, 2020.

3. 1 Page Research Poster Paper "Dynamic Modelling and Simulation of the Hypoxia-Inducible Factor 1 α (HIF-1α) SUMOylation in Response to Hypoxia" in the *43rd Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC2021)*.

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Chapter 1

Introduction

1.1 Background and Motivation

In recent years, the need to gain insight into the sensing, responding, and defending mechanisms for cells to extreme environmental and pathological conditions has increased rapidly. During tissue homeostasis, cells' growth and death rates are generally in equilibrium. However, the physiological homeostasis will be broken, and cells are in danger when exposed to environmental stresses. Over a long period of the evolutionary process, mechanisms have been developed within organisms to cope with these proteotoxic stresses in order to survive. In essence, if stress stimuli are too intense or persistent, consequently lacking inadequate time for recovery to normal condition, cells activate the death signalling process [1]. Accumulated studies have shown that stress-induced cell death is involved in varying human diseases, including neurodegenerative diseases and cancer [2]. Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) are incurable and debilitating conditions. The annual UK cancer and diseases diagnosis and treatment costs are enormous. Taking cancer as an example, the cost of diagnosing and treating cancer in the UK is expected to increase from £9.4 billion in 2010 to £15.3 billion by 2021, a rise of £5.9 billion or 62%, according to PharmaTimes 2021 report. In addition, Alzheimer's disease has been predicted to costs the economy £17 billion a year. Therefore, understanding how cell fate is determined in human diseases associated with such cellular stresses is vital.

One of such stresses that represents a significant obstacle to life [3–5] is the heat shock

(HS), which can be triggered by a temperature increase of only a few degrees (°C), and it has been observed that the relationships among sensitivity to small changes in temperature, exposure duration, and damage extent are very complicated [3][6, 7]. It is now well known that, in response to heat shock, cells activate an ancient signalling pathway leading to the transient expression of heat shock proteins (HSPs), helping to protect the repairing of damaged proteins within the cells [7, 8]. In the case of HS, HSPs serve as molecular chaperones assisting in the refolding of denatured or damaged proteins, while the upregulation of HSPs is through the gene expression of a specific group of transcription factors upon HS, which are known as heat shock factors (HSFs) [9, 10]. In particular, HSF1 is regarded as a critical regulator in the HSF family due to its ability to bind the heat shock element (HSE) in the HSP promoters region of the heat shock genes to regulate transcriptional expressions of heat shock genes to promote the expression of HSPs. The rapid induction of heat shock genes, which occurs in all organisms when they are exposed to high temperatures, has been extensively researched. Moreover, early experiments of heat shock response have been examined in [11–14] including HSPs transcription and HSF binding activity that expression of genes decreases upon a return to recovery.

Since Peper *et al.* released their seminal work on heat shock response (HSR) mathematical modelling, there has been some improvement in comparison to experimental investigations [4][15–18]. The computational results of the models [18] showed that, for the administration of the HS, a significant rise in HSP synthesis was initially observed and followed by a slight decrease back to its original steady-state value. In the case of a short HS, the same significant rise in HSP synthesis was initially observed as in the case of a long HS, but the subsequent decrease in HSP synthesis was faster than a long HS. A data-driven dynamical model of the HSR in Chlamydomonas reinhardtii was proposed in [16], in which a Monte Carlo scan of the parameter space was performed to gain insight into its structure, followed by a gradient search used to find a set of parameters that locally optimises the difference between the model simulations and experimental findings collected from the literature. All of these HSR models give some level of insight into the HSR mechanism.

Oxygen has a central role in the maintenance of life; in order to maintain oxygen homeostasis, advanced eukaryotes have adopted unique mechanisms to enhance O_2 absorption and distribution. Hypoxia, or oxygen inadequacy, triggers plenty of metabolic changes which

have rapid and profound consequences on cell physiology. The mechanism of the hypoxia response network has been proposed [19–21], where the critical factors, Hypoxia-inducible Factors (HIFs), are the head regulators of hypoxia-induced gene transcription [22]. Each HIF is a heterodimeric transcription factor complex consisting of one of the three different members (HIF1α, HIF2α, HIF3α), a common constitutive aryl hydrocarbon receptor nuclear translocator (ARNT) subunit, which is also known as HIF1β. HIF1α, in particular, is recognised as a critical regulator in the HIF family due to its ability to bind to the HIF1β and then relocate to the hypoxia response element (HRE) in the hypoxia genes. The expression level of hypoxia genes was regulated by the occupancy of HRE. prolyl hydroxylases (PHDs) act as a regulator of HIF, which covalently modifying the HIF1α subunits by hydroxylating HIF1α through direct perception of oxygen [23, 24]. The hydroxylated HIF1α subsequently interacts with the von Hippel-Lindau (VHL), tumour-suppressor protein, for degradation.

Kohn *et al.* [19] pioneered the mathematical modelling of the HIF pathway, as well as the creation of molecular interaction maps (MIMs). Since then, several mathematical models of HIF regulatory network have been proposed [19][23][25, 26], which focus on four topics: (1) oxygen-mediated switch-like behaviour of HIF nuclear accumulation and transcription activity; (2) HIF sensitivity to the molecular microenvironment; (3) role of Factor-inhibitors of Hypoxia (FIH) in the modulation of HIF activity and stability response; and (4) temporal dynamics of the HIF response to hypoxia. The results of the models [27] indicated that the HIF1α response increases exponentially with decreasing oxygen tension in the physiological range but decreases slightly at low oxygen levels (0.5% to 0%). A pathway of switch-like behaviour for HIF has been proposed in [19], in which the numerical integration of the ordinary differential equations (ODEs) system was executed to simulate the HIF1α protein stability and activity. These models provide some degree of new insights into the molecular mechanisms connecting hypoxia to the gene expression of cells.

Only recently, it has been understood that SUMOylation, a protein post-translational modifications (PTMs), is a key determinant of cell fate in response to environmental stresses, including heat shock and hypoxia [9][28, 29]. Small Ubiquitin-like MOdifiers (SUMOs) are a class of small proteins that can be covalently attached to specific eukaryotic protein targets in a cell process known as SUMOylation. SUMOs can be found in all eukaryotes,

such as yeast, plants and animals, but does not exist in bacteria and single-celled organisms [30]. In higher eukaryotes such as mammals, there are three conjugatable SUMO forms, SUMO-1, SUMO-2 and SUMO-3 [31]. SUMO-2 and SUMO-3 have a similarity of up to 95% in sequence and function; thus, they do not distinguish from each other, called SUMO2/3, which are significantly different from SUMO-1. SUMOylation is also a highly dynamic and reversible process, where Sentrin-Specific Proteases (SENPs) are involved in the deSUMOylation process. Recent studies have reported that several primary factors, such as heat shock factor (HSF), heat shock proteins (HSPs) and hypoxia-inducible factor (HIF), are associated with SUMOylation-dependent mechanisms in cellular stresses caused by heat shock or hypoxia [32, 33]. However, how SUMOylation affects HSF1 and HIF activity at a molecular level is still unclear. For instance, Low oxygen levels have been shown to promote SUMO modification, although the mechanism has remained a mystery [22]. It has been implicated in the regulation of a host of cellular processes and is essential for the health, and even the survival, of most organisms [30]. In other words, SUMOylation triggered by cellular stresses such as heat shock or hypoxia could be a survival mechanism for cells in response to various stimuli to survive. Moreover, it has been revealed that dysregulation of SUMOylation is related to a variety of disorders, such as cerebral ischemia, diabetes, cancer and neurodegenerative diseases [34]. Therefore, a better understanding of the SUMOylation regulatory machinery would lead to the development of new therapeutic strategies for preventing and/or treating those diseases associated with cellular stress.

As the new experimental evidence reported recently, it is important to update these existing models by augmenting them with PTM processes, in particular, the SUMOylation of HSF1. SUMO-2/3 conjugation to proteins is an essential component of the heat shock stress response, and it is proposed that increased SUMO-2/3 conjugation plays an important role in cell survival by assisting in the maintenance of protein complex homeostasis [35, 36]. When human cells are exposed to HS, in comparison to that in control cells, the majority of SENPs are inactivated, and that inactivation of SENPs seems to be an irreversible process [37], which further suggests that SUMO-2/3 conjugation is activated in response to HS. In addition, there have also been reports of hypoxia-inducible SUMOylation [38–41]. The importance of SUMOylation targeting factors of the hypoxia signalling process will be emphasized by the SUMOylation that is involved in cellular adaptation to hypoxia [42]. The regulation of SUMO-1 expression for hypoxia response in the brain and heart

of adult mice has been reported in [38], which a substantial increase in SUMO-1 mRNA and proteins after hypoxic stimulation in vivo. The initial research into the function of hypoxia in the regulation of protein SUMOylation discovered HIF1α to be a target for SUMOylation [38, 39]. The results in [39] mentioned that the SUMO-1 expression improved the stability and HIF1α transcription activity. Furthermore, it is well recognized that E3 ligase is an important regulator for protein SUMOylation [40, 41]. PIASy as a specific ligase enhanced hypoxia-induced HIF1α SUMOylation, while SENP1 removes SUMO from SUMOylated HIF1α to stabilise HIF1α, allowing for HIF1α to participate in the regulation of hypoxia signalling [43, 44].

Although the considerable progress of SUMOylation in response to metabolic and environmental stresses has been achieved over the past years, much more work is required to better understand the regulation, targets, and dynamic behaviour of SUMOylation and deSUMOylation in response to metabolic stresses. To overcome these challenges, the thesis focuses on developing data-driven modelling simulation, and sensitivity analysis of SUMOylation in response to heat shock and hypoxia, by combining methods from systems theory with theoretical and experimental techniques from molecular cell biology and biochemistry.

1.2 Overview of the Thesis

1.2.1 Aims and Objectives

The overall scope of this project is to develop advanced modelling, simulation and analysis methods for studying the complex SUMOylation and deSUMOylation regulatory mechanisms in response to extreme cell stresses, focusing on heat shock and hypoxia. By quantifying the signal pathways of sense, response, and defence to the extreme environment and pathological conditions observed in the experimental/clinical data, a detailed investigation of the underlying regulatory networks can be achieved.

The key objectives of the project are summarised as follows:

1. Investigate the SUMOylation and deSUMOylation process systematically and the underlying mechanisms of the external stress-response, including heat shock and hypoxia, signalling network to establish a mathematical characterisation.

2. Develop a mathematical model of SUMOylation-dependent collaboration of cellular stresses response, which can provide a physiologically relevant interpretation of the estimated parameters to describe the regulatory mechanism.

3. Validate the developed models by conducting independent experiments within a specific environment and pathological conditions. Furthermore, results that have not been experimentally verified can also be simulated with models.

1.2.2 Contributions of PhD Study

The main contributions of this thesis are listed below:

1. Mathematical models in combination with experimental validation for studying cell response to heat shock (HS) and hypoxia are developed, respectively. The author has focused on the mechanism of SUMOylation of the heat shock transcription factor-1 (HSF1) and hypoxia-inducible factor-1α (HIF1α). Key features of the HS model are the inclusion of heat shock response (HSR) and SUMOylation of HSF1, and heat shock protein (HSP) synthesis at a molecular level, describing the dynamical evolution of the critical variables involved in the regulation of HSFs. In the hypoxia model, similarly to the HS model, the primary aspects are the hypoxic response and the SUMOylation and hydroxylation of HIF1α, explaining the dynamic regulation of the key components associated with the stabilisation HIFs. The parameters of the model are optimised by using a differential evolution algorithm (DE) and genetic algorithm (GA). The research into cellular heat shock response (HSR) and hypoxia response using mathematical models has been reported over the past decades [3, 4][15, 16][19][23][26]. However, the role of SUMOylation in the heat shock response and hypoxia response has never been proposed by a mathematical model. To the best of my knowledge, this is the first attempt to investigate the response to heat shock and hypoxia associated with SUMOylation of HSF1 and HIF1α by combining experiment and mathematical modelling individually.

2. In the HS model, a differential evolution algorithm (DE) based data-driven modelling of SUMOylation-dependent synergism of hypoxia response on gaining a quantitative understanding of how HSF1 interacts with SUMO proteins to sense elevated tempera-

ture and respond to heat shock is presented. In the hypoxia model, on the other hand, a genetic algorithm (GA) based data-driven modelling of SUMOylation-dependent synergism of hypoxia response on gaining a quantitative understanding of how HIF1 interacts with SUMO proteins to sense oxygen and respond to hypoxia is also displayed. The model contains ordinary differential equations (ODEs) describing the dynamical evolution of the key variables involved in the signalling pathway, including temperature, HSF1, SUMO proteins and associated complexes in the HS model, while the oxygen tension, HIF1, SUMO proteins and related processes are used in the hypoxia model. The model parameters are optimised by applying the GA and DE based data-driven methods, and the results show that approaches have the potential for the modelling purpose. It is currently understood that the mechanism of SUMOylation in both heat shock response and hypoxia response is presented by mathematical modelling techniques for the first time in this study.

3. In this project, biomedical rate reactions are converted into an ODEs system describing potential biological processes using the law of mass action and Michaelis-Menten kinetics. In addition, in order to validate the heat shock model, experiments are performed to generate time-course data of the heat shock response of the cell. It has been shown that the predicted level of SUMO conjugation is in excellent agreement with experimental data. For hypoxia, there are very few time-course data available in the literature for the SUMOylation of HIF1. However, the developed mathematical model has been validated by the experimental data of time courses in the literature [43]. Furthermore, experimentally, attractive conditions that have not yet been tested can be simulated and predicted by employing the mathematical model.

1.2.3 Impact of the Work on the Scientific Community and Society

The successful implementation of this study will make a significant contribution to the research and treatment of human diseases. SUMOylation is an essential reversible dynamic process in eukaryotes. When cells are subjected to external stress, some protein-disordered diseases may be induced. Whereas SUMOylation will respond to the stimulus to reduce protein disorders and protect the cells from damage. It has been shown that SUMOylation is implicated in some diseases; for example, the level of SUMO-1 protein reduced in the

patient, which results from the mutated copy of SUMO-1 gene encoding [30]. In addition, misregulation of SUMOylation leads to tumorigenesis, and SUMO conjugation enzyme (E2) binding enzymes are overexpressed in human malignancies [30]. Furthermore, some experimental evidence in mice revealed that heat shock factor 1 (HSF1) could regulate and prolong the lifespan as a longevity factor, and it also proved that the deletion of the HSF1 gene might affect the spectrum of tumours [45]. Experimental studies conducted on mice have shown that heat shock-induced SUMOylation is associated with specific diseases and cancers, such as Alzheimer's disease (AD), lung cancer and breast cancer [46, 47]. Meanwhile, scientific and clinical evidence has indicated that modulating HIF1 may help reduce cellular and tissue damage in neurodegenerative diseases. HIF1 could be a viable therapeutic target for neurodegenerative diseases, according to these recent studies [48]. These can also serve as the basis for further research on diseases in humans or other mammals. The establishment and validation of a novel mathematical model would provide important insights into the dynamic processes of SUMOylation/deSUMOylation to better understand their relevance to health and diseased conditions. This would lead to some novel treatment or treatments interventions to be applied in hospitals to treat patients in the future.

In summary, an expanded modelling framework that allows inadequately characterised mechanistic models to be supported by data-driven methods is the ideal comprehensive healthcare modelling environment. By increasing the modelling tools at researchers' disposal, the range of tractable problems enlarges to encompass many remaining domain challenges and significantly enhances biomedical science. Moreover, given that cell stress-related diseases are increasing, for example, annual UK stroke incidence is over 150,000, with over 1.2 million stroke survivors, most suffering from neuronal dysfunction, the project outcomes will lead to a better understanding of these disease processes, more effective therapies, an enhancement in the quality of life of patients and carers, and an easing of the substantial economic and social burden on the UK healthcare sector.

1.2.4 Thesis Structure

This thesis is organised as follows:

In Chapter 1, the introduction of the thesis is presented. This chapter begins with the

background to and motivation for the project, followed by an overview of the thesis, including aims and objectives, the contribution of the PhD study, organisation of chapters and publications and presentations.

Chapter 2 reviews the cellular response to different stresses, including heat shock and hypoxia. Moreover, other stresses, such as ischemia and oxidative stresses, are discussed.

Chapter 3 explores the role of SUMOylation in the cellular stress response. The concept between post-translational modifications (PTMs) and SUMOylation is displayed. The role of SUMOylation in the cellular response to heat shock and hypoxia is presented in the next section. Furthermore, the association between SUMOylation and human diseases is mentioned.

Chapter 4 describes the dynamic modelling of the cellular response to heat shock. According to the biological mechanism of heat shock, the mathematical model of SUMOylation-dependent synergism of heat shock response is developed. To refine the value of parameters, the optimisation methods are described as the methodology. Meanwhile, experimental data were adopted to validate the model, these are consistent with the simulation results.

Similarly to the heat shock model mentioned in Chapter 4, the dynamic modelling of hypoxia is shown in Chapter 5. The biological process and mathematical model of the interplay between SUMOylation and hypoxia response are introduced firstly. After performing the optimisation algorithm, experimental data were collected for model validation. Due to the limited data, data augmentation was necessary to extend the amount of data to make the model validation more reliable.

MATLAB is the primary programming tool used in the project to achieve algorithms, such as genetic algorithm, differential evolution algorithm and global sensitivity analysis, and generate these results. The mathematical model was conducted by SIMULINK. Besides, Image J and Image Studio Lite are also used to analyse the experiment data and convert data into numerical value form.

Finally, in Chapter 6, a summary of the key findings of these research studies, conclusions and suggestions for further work are presented.

Chapter 2

Cellular Response to Different Stresses

2.1 Cellular Response to Heat Shock

2.1.1 Introduction

One of the most critical questions in cell biology is determining cell fate when exposed to extreme stresses such as heat shock. It has been long understood that organisms exposed to high temperature stresses typically protect themselves with a heat shock response (HSR), where an accumulation of denatured or unfolded proteins triggers the synthesis of heat shock proteins (HSPs) through the heat shock transcription factor, *e.g.*, heat shock factor 1 (HSF1). The relationship between deoxyribonucleic acid (DNA) transcription and ribonucleic acid (RNA) translation displayed in Figure 2.1.

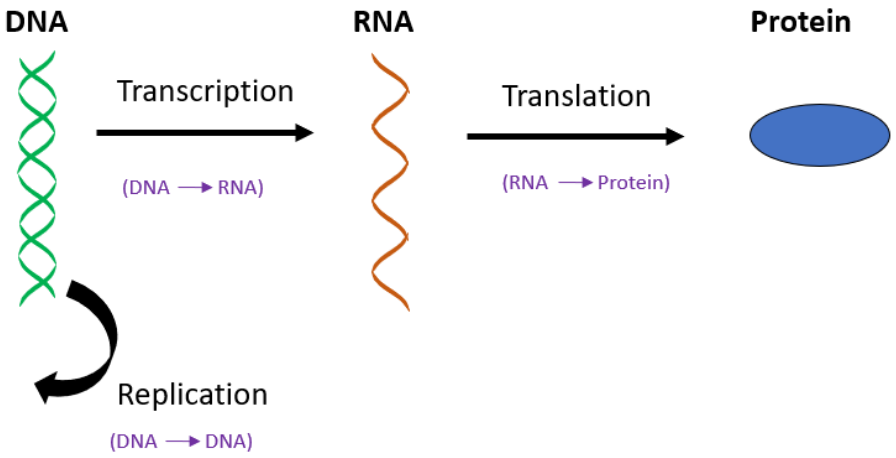

Figure 2.1. DNA replication, transcription and translation

Understanding how organisms react to extreme environmental stresses and/or pathological conditions is of crucial importance in developing fundamental metabolic therapeutic approaches to improving their survival under these conditions. One of such stresses that represent a significant obstacle to life [3–5] is HS, which can be triggered by a temperature increase of only a few degrees (°C). It has been observed that the relationships among sensitivity to small changes in temperature, exposure duration, and damage extent are very complicated [3][6, 7]. It is now well known that, in response to HS, cells activate signalling pathways leading to the transient expression of heat shock proteins (HSPs) [7]. HSPs are a large family of proteins and encoded by genes whose expression will be up-regulated when the cells are subjected to stress conditions, such as heat shock, oxidative stress, fever or inflammation, the presence of alcohol, inhibitors of energy metabolism and heavy metals, by helping to protect repairing the damaged proteins within the cells [7][49, 50]. Under normal physiological conditions, HSPs have multiple "housekeeping functions" in cells, in particular, as chaperones to assist the conformational folding or unfolding and the assembly or disassembly of other macromolecular structures [4]. In the case of HS, HSPs serve as molecular chaperones assisting in the refolding of denatured or damaged proteins, while the upregulation of HSPs is through the gene expression of a specific group of transcription factors upon heat shock, which are known as heat shock factors (HSFs) [9, 10]. In particular, HSF1 is regarded as a critical regulator in the HSF family due to its ability to bind the HSE in the HSP promoters region of the heat shock genes to regulate transcriptional expressions of heat shock genes to promote the expression of HSPs.

HSP70s are the most extensively studied group of stress proteins among six groups and can be identified in all major cellular compartments of eukaryotes to date [51]. As one of the most highly conserved groups of proteins of molecular chaperones in the biological world [7], the genes encoding the HSP70 are highly conserved as well. The expression of HSP70 is involved in the normal physiological processes of cells and in response to or defence of cells to external environmental changes or stimuli. Moreover, HSP70 plays a core role in a large number of protein-folding processes. For example, they assist in the correct folding of proteins under stress conditions, and in preventing the aggregation of unfolding proteins and then refolding or degradation of aggregated, and denatured proteins [52, 53]. In addition, another function of HSP70 is that they support the transmembrane translocation

of proteins [54, 55]. Three activities are documented that according to the role of HSP70 in the folding of non-native proteins: prevent aggregation, facilitate proteins-folding into a native state and finally, gather and refold aggregated proteins [52].

2.1.2 Molecular Chaperones and Proteins Folding

Functional proteins in cells usually exist in their natural, completely folded state, most of which must be folded into predetermined three-dimensional structures to have functional activity. However, protein synthesis and translocation in the cell are both critical processes in protein biogenesis, requiring that proteins reside in an unfolded or partly folded state for some time. As a result, portions of a polypeptide that are normally hidden in its natural conformation become revealed and interact with other proteins, producing protein aggregation and the production of potentially hazardous species. This suggests that freshly synthesized proteins are vulnerable to abnormal folding and aggregation in the cellular environment, which is harmful to the cell [56, 57]. In order to survive, the cells acquired a mechanism called chaperones to protect nascent proteins during folding in order to prevent aggregation when the proteins unfolded. It is revealed that over half of all mammalian proteins are folded by chaperone proteins, which are involved in the folding of thousands of distinct polypeptides into a variety of forms. At the organismal level, chaperones govern the proteome in both the intracellular and extracellular environments [58]. Research over the past decades has established that molecular chaperones are essential in preventing protein aggregation and helping proteins to fold correctly at the molecular level [57][59, 60].
In vivo, molecular chaperones promote the folding of proteins and the construction of multi-aggregated structures, which is not a wholly spontaneous process [51]. Molecular chaperone initially refers to a nuclear protein that binds to histones in the nucleus and mediates the orderly assembly of nucleosomes [61]. Now a the concept of molecular chaperones has been expanded to define a class of proteins that are unrelated in sequence but have a standard function. In a non-covalent manner, molecular chaperones bind to nascent polypeptides and unfolded or unassembled proteins, assisting protein synthesis in two ways. In other words, molecular chaperones help other polypeptide-containing structures in the cell to self-assemble then adequately separate from each other after assembly, which does not constitute a component of the function of these protein structures.

As biochemists have said that a molecular chaperone is “any protein that binds another protein and has no function of its own” [61, 62]. In general, molecular chaperones are a class of proteins that recognize and bind to proteins that are folding incompletely or assembled, helping these proteins fold correctly, transport or prevent aggregation, and meanwhile are not involved in the formation of the final product.

Molecular chaperones can acts as “holdases” to stabilize the formation of non-native proteins, as “foldases” to assist folded-proteins to native-proteins or as a “unfoldases” to unfold misfolded-proteins [59][63]. Molecular chaperones are a vital part of each of the aspects of the protein homeostasis network (PN), including protein folding, refolding, degradation, and synthesis [64]. Protein folding is the process through which a protein acquires its functional structure and confirmation. Through this physical process, proteins are folded from the random curly structure into the specific functional three-dimensional structure to ensure they function normally. For freshly synthesized proteins, the risk of misfolding and aggregation is high in the cellular condition. Therefore, based on the potential risk, molecular chaperones are applied to prevent aggregation and ensure the folding is performed correctly. Protein homeostasis (proteostasis) is widely known for its significance in sustaining the function of the proteome. Proteostasis is disrupted by genetic mutations for ageing, resulting in improperly folded proteins that frequently lose function. The accumulation of misfolded and aggregated proteins are also cytotoxic and have been linked to the development of neurodegenerative diseases [60]. It has been shown that the molecular chaperone is associated with neurodegenerative diseases and that mutations in it lead to neurodegenerative diseases [65], which further underscores the role of molecular chaperones in preserving neuronal protein stability by identifying mutations in molecular chaperones in familial cases of neurodegenerative diseases. Increasing the expression of molecular chaperones can reduce protein aggregation and toxicity in a variety of neurodegenerative disease models, making molecular chaperones promising therapeutic targets [65, 66].

2.1.3 Major Classes of Molecular Chaperones—Heat Shock Proteins

After discussing the notion of molecular chaperones, it is time to consider why the expression of particular chaperones is elevated in cells under metabolic stress. Organisms

must undergo a variety of stressful conditions, including rapid temperature increases that harm crucial cellular structures and disrupt essential functions. Cells activate an ancient signalling pathway, which induces the activation of a group of genes known as heat shock proteins (HSPs) [7][67, 68]. Many environmental stressors such as heavy metals, ethanol, amino acid analogues, and hypoxia have induced similar reactions in studies. HSPs, therefore, are also known as stress-inducible proteins.

Stress-inducible proteins are classified into seven categorizes based on their function. The first identified HSPs, now widely referred to as "molecular chaperones", are the most prevalent class across species in terms of expression level [69]. Components of the proteolytic system, which are required to eliminate misfolded and permanently aggregated proteins from the cell, belong to the second class. The third category is concerned with the treatment of non-physiological covalent alterations of nucleic acids. This group of enzymes comprises RNA- and DNA-modifying enzymes, which are necessary to repair DNA damage and processing errors that arise under stressful situations [70]. Metabolic enzymes make up the fourth HSP group. The tremendous significant variance across species may be seen here. Although there are few systems methods for modelling metabolic pathways during and after stress, evidence suggests that modifications in pathways may be essential to restructure and stabilise the cell's energy supply [71, 72]. The fifth class of proteins compares transcription factors and kinases, some of which are required to begin stress response pathways further or block expression cascades, such as ribosome assembly routes [73]. Proteins that keep cellular structures such as the cytoskeleton in place belong to the sixth class. Finally, transport, detoxifying, and membrane-modulating proteins are among the elevated proteins in the seventh group [74].

HSPs have complicated protective mechanisms, and the most conserved HSPs are molecular that aid in the formation of natural protein structures by inhibiting the formation of nonspecific protein aggregation [7]. The majority of HSPs are involved in various aspects of protein biogenesis and are ubiquitous proteins found in the cells of all studied organisms [75].

Exposing cells to dramatically increased temperature transiently induces the expression of HSPs [45]. However, many other environmental and metabolic stresses may also trigger HSPs expression, including heavy metals, anoxia/hypoxia, nicotine, ethanol, surgical stress and ischaemia. HSPs are general proteins in various tissues and cells of every

species. Meanwhile, accumulated pieces of evidence show that HSPs play a key role in cell stress response and normal physiological functions [8]. Various cellular processes such as synaptic transmission, autophagy, endoplasmic reticulum (ER) stress response, protein kinase and cell death signalling are associated with increased HSPs expression. The state of neurological injury and diseased cells is significantly linked to HSPs expression [8]. Neurodegenerative disorders are associated with increased expression of misfolded proteins, which are known to trigger some degenerative diseases, including Huntington's disease (HD), Kennedy disease (KD), Parkinson's disease (PD), and Alzheimer's disease (AD) [76]. HSPs assist in assembling, protein folding and translocating. Apart from these functions, HSPs play a role in protecting cells against injuries, and other various stresses [77].

Molecular chaperones of mammalian species are classified into six major groups according to their molecular weight and homology, such as small HSPs (15–30 kDa) including HSP27, HSP40s ($\approx$ 40 kDa), HSP60s ($\approx$ 60 kDa), HSP70s ($\approx$ 70 kDa), HSP90s ($\approx$ 90 kDa) and HSP100s ($\approx$ 100 kDa) [49][64][68]. Each group has multiple members with identical sequences and functional domains, and their tissue expression occurs in distinct subcellular compartments and to varying degrees. HSP70 is the one that is most strongly triggered by increased temperature among the several HSPs. As a result, it was the chaperone of significance to investigate in the setting of hyperthermia [78]. HSP70, with a molecular weight of 70kDa, is the most conserved molecular chaperone in all living organisms and the genes encoding for that protein. HSP70 is a chaperone that accumulates in response to cellular various stressors, facilitating cell survival. HSP70 is distributed throughout various cellular compartments such as in the cell membranes of bacteria, archaea and eukaryotes, as well as in the nucleus, mitochondria, chloroplasts, and endoplasmic reticulum (ER) of eukaryotes [79, 80]. DnaK, the prokaryotic counterpart, has roughly 60% sequence similarity to eukaryotic HSP70 proteins present in the cytoplasm and organelles such as the ER, mitochondria, and chloroplasts. HSP70s are associated with the de novo folding of proteins under normal conditions, and under stressful environments, they prohibit unfolded proteins from aggregating and can even refold aggregated proteins [52]. It has been indicated that HSP70 controls a broad range of biological functions, including polyetide folding, degradation and translocation across membranes, and protein-protein interactions [81]. There are three types of non-native protein folding regulated by

HSP70: aggregation inhibition, native folding elevation, and solubility and refolding of aggregated proteins [52][82]. In addition to these functions, it is becoming more obvious that HSP70 is regulated by transcriptional and post-translational modifications, including nucleotide-binding, carbonylation, phosphorylation, oligomerisation [8]. HSP70 comprises a highly conserved N-terminal ATP binding domain (NBD) of 45 kDa, which has a weak ATPase activity that can be triggered by binding to unfolded proteins and synthetic peptides, and a more variable C-terminal substrate-binding domain (SBD) of nearly 25 kDa, which is composed of a 15 kDa-sandwich subdomain and a C-terminal α-helical [68][82]. The latter identifies the hydrophobic amino acids found in proteins. In particular, the exposure of this patch of hydrophobic amino acids is linked to a protein's inclination to assemble. The HSP70 helical lid is open when ATP is bound, and it can be only be closed when ATP hydrolysis occurs [58][83]. The HSP70, like other chaperones, does not recognize a consensus sequence motif. In an extended conformation, the binding site holds a length of seven amino acids, most of which are hydrophobic [84]. In the posthydrolysis ADP state, HSP70 contacts substrates with significant affinity, indicating that its interaction with unstructured polypeptide segments is ATP dependent shown by Alberts *et al.* [85] in Figure 2.2. Cofactors control the activity of HSP70, and the HSP40-containing proteome is the largest class of HSP70 cofactors, attaching to non-native proteins and transporting them to HSP70 [7][81].

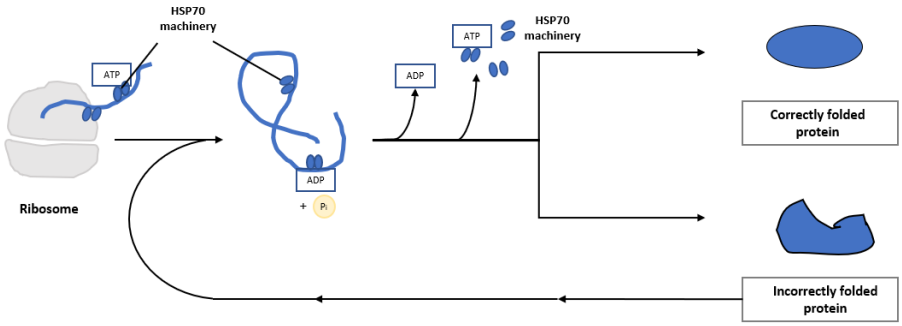

Figure 2.2. The Function of HSP70 in Protein Folding. Adapted from [85]

In addition, the formation of denatured protein aggregates due to misfolding challenges the PN and increases the possibility of developing the disease. Indeed, abnormal levels of stress proteins are associated with many diseases such as heart failure, fever, infection, ageing, Alzheimer's disease, malignant disease and autoimmune diseases [77][86]. Emerging evidence proposes that manipulation of HSP70 has a significant impact on cell fate

in neurological damage and diseases. Furthermore, there is much evidence that HSP70 is overexpressed in cancer and that high expression of this chaperone is related to a higher tumour grade and a worse prognosis [87, 88]. Overexpression of HSP70, for example, is a sign of liver and prostate cancer in its early stages [89, 90]. In colorectal carcinoma and breast cancer, overexpression of this protein indicates advanced diseases, and lymph node metastases [91].

A 27kDa protein called HSP27, whose expression is observed to correspond with enhanced survival in response to cytotoxic stimuli is one such protein that is highly elevated during the stress response. It has been found to protect cells against a range of apoptosis-inducing stimuli. HSP27 is a member of the small heat shock proteins (sHSPs) family of abundant and ubiquitous stress proteins found in practically all organisms, from prokaryotes to mammals [92]. HSP27 works as a molecular chaperone to help non-native proteins refold. It forms complexes with these proteins, limiting nonspecific aggregation and allowing them to be returned to their natural structure in the presence of ATP-dependent chaperones such as HSP70 [93, 94]. As opposed to other HSPs, HSP27 have an ATP-independent chaperone capacity. The molecular chaperone activity of this family is controlled by the C-terminal of sHSPs [95, 96].

HSP27 levels are generally low in unstressed cells, and HSP27 occurs primarily as a sizeable oligomeric unit of up to 800 kDa, usually made up of six trimeric complexes of the protein. Temperature, pH, ionic strength, and the degree of phosphorylation of the individual monomers all influence the size of the monomers. A rise in HSP27 expression is followed by a phosphorylation-induced rearrangement of the protein's multimeric state during the stress response [97]. Furthermore, HSP27 regulated apoptosis, according to new findings, is by interacting with significant components of the apoptotic signalling system, including those implicated in caspase activation and apoptosis [97]. HSP27 expression is undeniably a protective strategy for cellular survival during times of stress.

HSP40 belongs to a broad and understudied co-chaperone family. The human genome contains about 41 species of the HSP40 family. These individuals are thought to live in various intracellular locations [98]. HSP70 function is thought to be regulated by the HSP40 family, also known as chaperone DnaJ. The present understanding of the HSP40 family's relationship to human malignancies is limited and contentious. HSPs are overexpressed in a wide range of human malignancies, suggesting that they may give resistance to cytotoxic

therapy [99]. HSP40, HSP70 and HSP90 all had significant levels of expression in brain tumours, according to recent research. Furthermore, HSP40 was shown to be substantially expressed in lung cancer tissues in research. In addition, it has been discovered that anti-HSP antibodies might be used to identify HSP40 levels in cancer patients' serum, which can be employed to diagnose tumours. In humans, genome-wide research showed 41 DanJ-HSP40 family members that may be important for critical tasks, as previously noted. These proteins can be found in mitochondria, cytosol, ER, endosomes, nuclei, and ribosomes, among other intracellular locations [100].

In addition, the role of the HSP40 family in carcinogenesis and pathological events has become a topic of discussion in recent years. For example, some studies have suggested that members of the HSP40 family are involved in regulating tumour growth [100]. These insights are already helping to shape new, and potential clinical oncology roadmaps [101].

HSP60, a molecular chaperone protein with a molecular weight of 60 kDa, is most typically found in mitochondria and has a role in protein homeostasis. HSP60 may translocate to the cytosol and cell membrane in response to stress, as well as release HSP60 into the blood to generate serum HSP60 [102]. HSP60's capacity to respond to various stresses is highly reliant on its location [103]. HSP60 is recognized as one of the most crucial proteins for cell survival because of its critical involvement in mitochondrial function. Moreover, increasing evidence has shown that HSP60 and inflammation are associated. HSP60 is a robust regulator of innate immune cell activity, hence imparting substantial immunoregulatory effects that may have an impact on the development of inflammatory and autoimmune diseases, according to mounting data [104]. When produced on or secreted by autologous cells, extracellular HSPs have been shown to behave as potent intercellular signalling molecules that serve as danger signals to the innate immune system, imparting a broad spectrum of immunological responses [105, 106].

HSP90 is a molecular chaperone essential for the stability and function of some conditionally activated and/or expressed signalling proteins and several mutants, chimeric, or overexpressed signalling proteins that enhance cancer cell proliferation and survival or both. HSP90 inhibitors promote inactivation, instability, and eventual destruction of HSP90 client proteins by binding exclusively with a single molecular target, and they have demonstrated promising anticancer action in preclinical model systems. HSP90 inhibitors are unusual in that they disrupt numerous signalling pathways that typically

cooperate to enhance cancer cell survival while targeting a specific biological target. Under normal circumstances, HSP90 is found in large quantities in the cytoplasm of bacteria and eukaryotic cells, and it is elevated much more when they are stressed [107]. HSP90 as a molecular chaperones is prevalent protein in eukaryotic cells and may be present in most cellular compartments such as cell membranes, endoplasmic reticulum (ER), mitochondria and chloroplast [108]. The chaperone HSP90 is ATP-dependent and aids in the folding of many proteins, as well as the refolding of denatured proteins following stresses [109]. HSP90 binds multiple substrates in their natural forms and targets a specific set of signal transduction client proteins [110].

In some respects, this chaperone is unusual. Firstly, its substrate spectrum does not appear to be as diverse as HSP70 [111–113]. Secondly, it binds native-like proteins rather than unfolded proteins [114]. Thirdly, it appears to have evolved from a single constituent system in prokaryotes to the most complex chaperone machinery known in eukaryotes, collaborating with a huge cohort of co-chaperones that associate in a specific order during the chaperone cycle [115–117]. Only two of such co-chaperones, Sti1 and the prolyl isomerase Cpr6, are increased following stress in yeast, which is noteworthy in the context of stress management [118]. Sti1 is an uncompetitive inhibitor of the HSP90 ATPase that blocks additional conformational changes necessary for substrate processing while keeping HSP90 in a configuration that may improve HSP90s' interaction with substrate proteins [119, 120]. Therefore, under heat shock circumstances, Sti1 may allow HSP90 to fulfil a more fundamental holding role, preventing unfolded protein aggregation. Whether the substrate spectrum of HSP90 alters during stress conditions remains unanswered. What happens to HSP90-bound substrates when physiological circumstances are restored is also unclear.

2.1.4 Role of HSPs in Cancer and Different Diseases Development

Cancer is one of the most severe public health issues, which is a primary cause of mortality and a significant impediment to extending life expectancy in the world [121]. According to the World Health Organization, 18.1 million new cancer cases and 9.6 million cancer deaths were reported in 2018, while it became the top or second major cause of death before the age of 70 in some countries in 2019 [122]. Cancer is increasingly recognized as a disease

that involves dysfunction of numerous pathways controlling fundamental cell activities, including death, proliferation, differentiation, and migration, in addition to being a very heterogeneous pathology in terms of cell type and tissue origin [123]. Surgical treatment, chemotherapy, radiation, and immunotherapy have all progressed significantly during the last several decades. Treatment resistance is conferred through treatment-induced cellular genetic, and metabolic alterations [124]. As a result, there is a rising demand for innovative therapeutic techniques and biological targets for cancer treatment. Much cancer biology research has identified a plethora of prospective cancer therapeutic targets—a molecular chaperone, which belongs to the heat shock protein family known as HSPs. HSPs have been found to have aberrant expression levels in a variety of malignancies, including prostate, bladder, breast, ovarian, colorectal and lung cancers [88]. In addition, there are many pieces of evidence demonstrating that HSPs are associated with various types of diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) [125].

As described previously, the mammalian HSPs are classified into six classes based on their molecular size: HSP100, HSP90, HSP70, HSP60, HSP40 and sHSPs. Among them, HSP90, HSP70 and HSP27 chaperones stand out as anticancer targets because they are critical moderators of several on co-proteins (especially their client proteins) and have been shown to have tumorigenic features [126].

It is proposed that HSP70 is overexpressed in human cancer, including prostate cancer, breast cancer, and lung cancer [87–91]. It is widely considered that increased HSP70 production in transformed cells is thought to protect these cells from apoptosis, aneuploidy-related stress, and mutant protein accumulation, as well as the proteotoxic stress associated with speedy proliferation [127]. Following the realisation that HSP70 is a promising anticancer target, various research groups concentrated on developing HSP70 inhibitors for cancer treatment [127, 128]. Furthermore, studies have provided evidence for the potential role of HSP70 in aggregate-related neurodegenerative diseases over the years. Besides, HSP70 associates transiently with Huntington aggregates, with association-dissociation properties identical to chaperone interactions with unfolded polypeptides. Alzheimer's disease (AD) is the most prevalent form of dementia and is defined as a neurodegenerative disorder in the elderly. High levels of HSP70 expression were found in afflicted areas of AD brain tissues, as shown in Figure 2.3, and these levels seemed to be connected to the

dysfunctional, or stressed neurons [129]. The HSP70 participates in AD via pathological and/or protective processes where such chaperones.
participate.

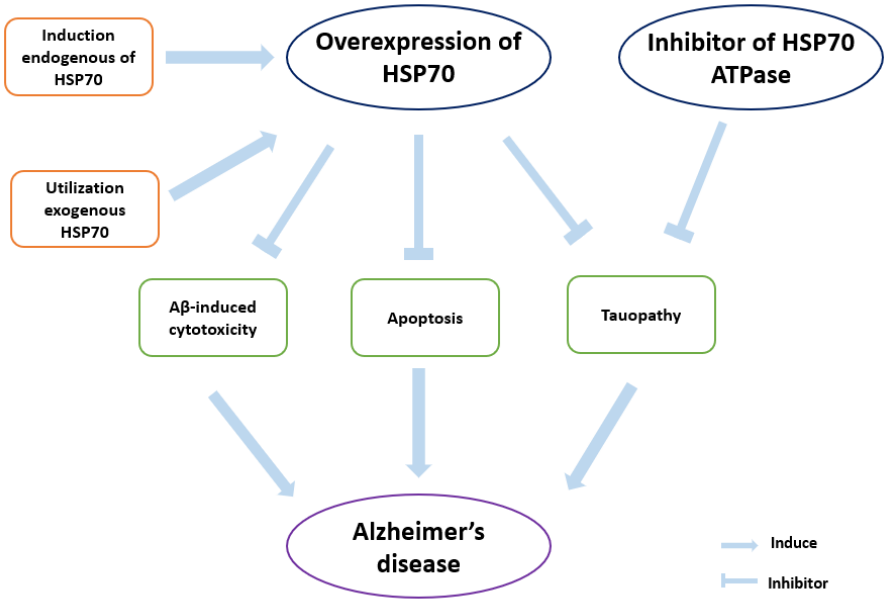

Figure 2.3. Expression of HSP70 in Alzheimer's Disease. Adapted from [125]

HSP90 have been referenced in differing human diseases, including cancer, neurodegenerative diseases and other diseases. The involvement of HSP90 in various diseases varies, is described below for some of the most frequent diseases related to HSP90. HSP90 is critical for cancer survival because cancer cells increasingly rely on HSP-assisted signalling pathways. HSP90 is overexpressed in cancer cells and is required for the malignant transformation and development of a variety of cancers, including bladder, breast, lung cancers, and also leukaemia [130]. Furthermore, tumour cells may become more reliant on HSP90 due to disruptive oncogenic alterations. Indeed, the levels of HSP90 are elevated dramatically in tumours, and overexpression of HSP90 is related to a poor prediction of breast cancer [131, 132]. Chemical inhibitors of HSP90 are frequently more responsive in cancer cells than in normal somatic cells. Tumour cells containing HSP90 in a multimolecular complex, may increase their affinity for the inhibitor [133, 134].
HSP90 was attributed to various neurodegenerative diseases, including AD and PD, which both involve protein aggregation as a feature. A role for HSP90 in the maintenance of neu-

rodegenerative diseases is thought to be similar to its proposed role in cancer: misfolding or stabilisation of aberrant (neurotoxic) client proteins. HSP90 clients include the fibril and plaque-forming protein amyloid-β (Aβ) and the hyperphosphorylated tau protein, both of which have been associated with Alzheimer's disease [135]. HSP90 is supposed to be able to target abnormal folds in both Aβ and tau to avoid aggregation [136, 137] displayed by campanella *et al.* [125] in Figure 2.4. HSP90 can be free or attached to exosomes in the extracellular environment. It has the effect of activating the immune system when it is outside the cell [138]. Therefore, upregulating HSP90 activity might be beneficial in treating these neurodegenerative diseases in certain circumstances. In Alzheimer's disease, on the other hand, inhibiting HSP90 may decrease the activity of kinases that hyperphosphorylated tau protein, reducing aggregation. Additionally, inhibiting HSP90 could lead to the degradation of abnormal disease-associated proteins [136]. In conclusion, HSP90 is beginning to be grasped as affecting neurodegenerative diseases, opening up the possibility of its future use as a potential therapeutic target.

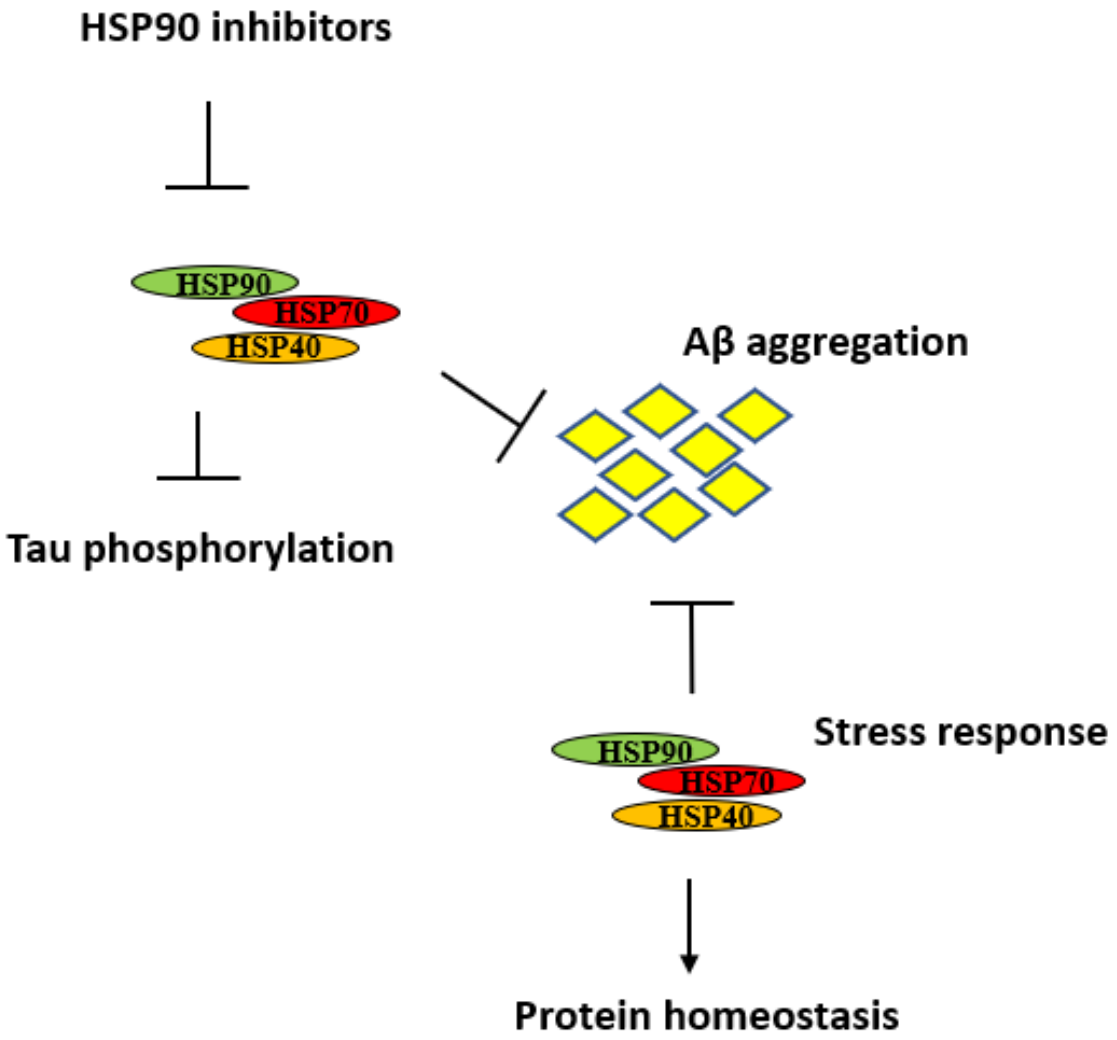

Figure 2.4. The role of HSP90 in diseases. Adapted from [125]

HSP27 is a small ATP-independent chaperone that protects against protein aggregation and can generate oligomers in response to stress, which is regulated by its phosphorylation status [139]. It processes antiapoptotic effects and increases production in response to stress [97]. This HSP has an unusually high basal expression in cancer cells, indicating that it is crucial for cancer detection [140]. This perplexing expression is also related to increased tumour pathogenicity, treatment resistance, and, as a result, a bad prognosis. By inhibiting this HSP, drug resistance can be addressed by increasing cellular sensitivity to medicines. Antisense oligonucleotides and small interfering siRNAs created to down-regulate HSP27 have been proven to have substantial anticancer action [141–143], and antisense oligonucleotides are already in clinical trials for a variety of human malignancies, including lung, breast, prostate, bladder, and ovarian cancers [144].

2.1.5 Regulation of Heat Shock Factors and the Heat Shock Response

The heat shock response (HSR) is one of the most ancient and evolutionarily most conserved defensive systems in nature. The HSR is a well-known way for cells and organisms to react when they are exposed to high temperatures, such as heat shock. Conditions that induce acute and chronic stress are constantly posed to living cells. Eukaryotic cells have evolved networks of varied reactions to detect and manage various sorts of stress in order to adapt to environmental changes and survive different types of injury [145].

The HSR is the rapid induction of HSPs in response to stress that occurs due to a range of genetic and physiological processes. The study of a universal protective mechanism that prokaryotic and eukaryotic cells use to maintain cellular function and homeostasis led to the discovery that a few degrees above the physiological temperature promotes the creation of a small number of proteins in *Drosophila* salivary glands [146].

The HSR is mediated at the transcriptional level by heat shock factors (HSFs), a group of transcription factors capable of specifically binding to heat shock elements within the promoter of HSP genes. HSF in higher eukaryotes is changed from a monomeric to a trimeric form in response to heat stress, allowing for high-affinity binding to both HSE and transcription factor [147]. The HSR process presented by westerheide *et al.* [86] in Figure 2.5.

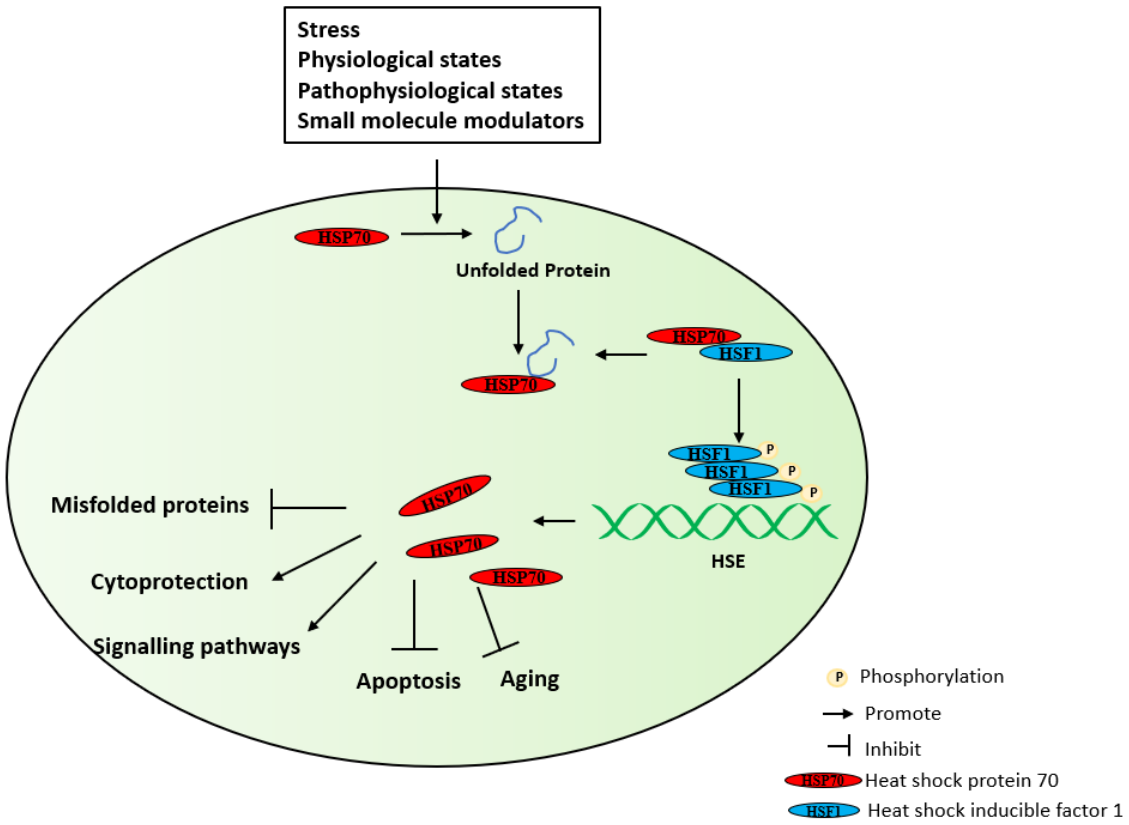

Figure 2.5. The process of heat shock response. Adapted from [86]

The transcription factor 1 (HSF1), which was first identified in cellular extracts from *Saccharomyces cerevisiae* and *Drosophila melanogaster*, oligomerises on activation and binds to heat shock element (HSE) sequences to control the stress-inducible expression of HSP genes. Whereas a single HSF exists in yeast and flies, three HSFs (HSF1, HSF2, and HSF4) were identified in mammals. HSF3, the fourth member of the HSF family, has been described in chickens but not mammals.

In eukaryotes, the expression of HSPs is increased upon various types of stresses, and the upregulation of HSPs requires the gene expression of a definite group of transcription factors for heat shock, which are known as heat shock factors (HSFs) [3][10]. When cells are attacked by harmful stresses, the native proteins are transformed into misfolded proteins, which could disturb normal cellular processes. However, cells need to take action to maintain the typical cellular environment, and heat shock response is a way against these harmful stresses [65][148]. HSFs are transcription factors that determine the efficiency of cellular heat shock response. Four members of the HSFs family are mediators of heat shock response, i.e. HSF1, HSF2, HSF3 and HSF4. HSF1 is regarded as a critical regulator

among HSFs families due to its ability to bind the HSE in HSPs' promoters region of heat shock genes [149]. Most of the HSFs need to form a binding between the DNA binding site and HSE when responding to some stresses, such as temperature rise, oxidants, heavy metals and bacterial viral infections, and regulate transcription of heat shock genes and promote expression of HSPs in the end. HSF1 is the primary regulator of the heat shock response, a well-conserved protective mechanism. Under a variety of pathophysiological circumstances, the HSF1 function improves survival. HSF1 can be found in unstressed and stressed cells. Under normal conditions, the HSF1 exists as a monomer form, which is an inactive form, and is unable to bind to HSE, HSP70 and other HSPs [14]. Under stressful conditions, such as elevated temperature, heavy metals and oxidants, HSF1 is activated by trimerisation and phosphorylation. After that, the binding of trimeric HSF1 and HSE is formed, and the function of transcription of heat shock genes is activated, which is why HSPs are accumulated. There are two functions of HSPs' accumulation: (1) enhancing a cell's power to prevent damage and eventually achieving the cell protection; (2) as a negative feedback regulating the cellular stress response through binding to HSF1 and inhibiting its continued activation [150]. It has been discovered that HSF1 is not only the vital regulator of heat shock response but also an essential player in responding and preventing proteotoxic, ageing and oncogenesis [151]. HSFs consist of functional domains. There are two conserved core areas: DNA binding domain (DBD) and trimerisation domain (TD). The DNA binding domain is close to the N-terminal of HSF1, which is located in the most conserved region. The specific binding site for the DNA binding to HSF1 is HSE. When HSF1 is activated, it is necessary to form an HSF1 trimer by combining the trimerisation regions with each other. Relative to the DNA binding domain, the trimerisation domain is located at the C-terminal of HSF1 [45].

The mammalian HSF family consists of four members: HSF1, HSF2, HSF3 and HSF4. HSF2 interacts with the promoters of HSP genes, while HSF1 is the primary regulator of the heat shock response. According to our existing knowledge, HSF2 relies entirely on the actions of HSF1 response to stresses since it is only inducted to gene promoters of HSP when HSF1 is present, and this collaboration necessitates an intact HSF1 DBD. Nonetheless, HSF2 affects the HSF1-mediated inducible expression of HSP genes positively and negatively, demonstrating that HSF2 can play a role in heat shock response transcriptional control.

2.1.6 Mathematical Modelling of Heat Shock Response

Compared with experimental studies of HSR, there has been some progress [4][16, 17] in its mathematical modelling since Peper *et al.* published their seminal work [15]. The computational results of the models [18] showed that, for the administration of the heat shock, a significant rise in HSP synthesis was initially observed and followed by a slow decrease back to its original steady-state value. In the case of slow heating, only a small rise in the synthesis of HSFs was observed, i.e., almost no changes in the original level. In the case of a short HS, the same significant rise in HSP synthesis of HSFs was initially observed as in the case of a long HS, but the subsequent decrease in HSP synthesis was faster than in a long case. In [16], Magin *et al* proposed a data-driven dynamical model of the heat shock response in *Chlamydomonas reinhardtii*, where a Monte Carlo (MC) scan of the parameter space was performed to gain insight into its structure, and then a gradient search was used to find a set of parameters, which locally optimises the difference between the model simulations and experimental data. The final model was validated with independent experimental results extracted from the literature for certain behaviours. All these HSR models provide some degrees of insight into the mechanism of the HSR.

As discussed earlier, HSPs play a core role in heat shock response, as a molecular chaperone, helping misfolded proteins (MFPs) to refold and ease the cytotoxic effects of damage [152]. The development of mathematical models has been improved through understanding the importance and complexities of the heat shock response. The first model of heat shock response, which did not include a thorough explanation of transcription regulation, has been developed in [15]. And then, the expression of HSPs and transcriptional regulation with more details has been determined [17].

The model in Peper *et al.*, [15] involves the dimeric and trimeric forms of HSFs, the heat-induced misfolded proteins and mRNA molecules interaction with HSP70. The model was verified by data acquired from experiments with H35 rat hepatoma cells. However, the disadvantage of this model is that the details of HSPs-regulated transcription are not considered. The model in Rieger *et al.* [17] studied the heat shock response of eukaryotes associated with HSPs, HSFs and HSE, as well as HSP-mRNA molecules, stimulation signals and kinases. This model refers to the process related to transcriptional activation of heat shock genes, including HSF1 binding DNA, phosphorylation of HSF1 and its tran-

scriptional activation. However, there are shortcoming of the model, as some essential and significant parts of the heat shock response are not mentioned, such as trimerisation prior to HSF1 binding to DNA, multiple phosphorylation reactions on HSF1 trimers and the degradation of HSPs. Although disadvantages are presented, it provides a comprehensive framework for the heat shock response of eukaryotes.

Moreover, except for the model in [15] and [17], different related models are proposed. The heat shock response model in [153] includes the basic biological reactions in the heat shock process, but misfolding of proteins is not taken into account, and only a part of the heat shock models are analysed in the analysis of mathematical models, which makes the results incomplete. A completely different modelling approach is established in [154]. The model uses the experimental data of heat shock response of Chinese hamster ovary cells as the beginning of modelling and creates a stochastic theoretical model while also considering the mean response of the cell population. Interestingly, the transactivation of HSFs-regulated HSPs-encoding genes was rediscovered in this way. In addition, there is a sensitivity analysis of the heat shock model [152]. In this model, the level of MFPs in the steady-state of the system is reduced by changing some parameters, which may make the cells more susceptible to the heat shock response.

2.2 Cellular Response to Hypoxia

2.2.1 Introduction

It is critical to understand how cell fate is determined when exposed to extreme stress encountered in both physiological processes and pathophysiological conditions. Oxygen has a central role in the maintenance of life, in order to maintain oxygen homeostasis, and advanced eukaryotes have adopted unique mechanisms to enhance O_2 absorption, and distribution [23][155, 156]. These mechanisms rely on modifying pre-existing proteins, translational arrest and transcriptional changes. Hypoxia initiates various of metabolic problems which have direct and far-reaching implications for cell physiology [23]. The transcriptional stimulation of a variety of genes involved in angiogenesis, iron metabolism, glucose metabolism, and cell proliferation/survival occurs when cells and organs adapt to low oxygen tension [157].

Oxygen is essential to the life of all living organisms. Almost every cell type may detect a lack of oxygen (hypoxia) and, as a result, activate a set of oxygen-regulated genes. Much progress has been made in our understanding of how hypoxia stimulates the expression of oxygen-regulated genes since the discovery of hypoxia-inducible factors (HIFs) [158]. Hypoxia-inducible factor 1 (HIF1), an oxygen-sensitive transcriptional activator, is the central mediator of this reaction. HIF1 is made up of two subunits: HIF1β, which is constitutively expressed, and HIF1α, which is activated by oxygen (or its analogues, HIF2α and HIF3α). Post-translational changes, including hydroxylation, ubiquitination, acetylation, and phosphorylation, influence the stability and function of the HIF subunit. HIF1 degradation is impeded under hypoxic circumstances, resulting in protein build-up, dimerisation with HIF1, binding to HRE inside target genes, and transcription activation through recruitment of cofactors p300 and CBP [159].

Evolutionarily conserved mechanisms are activated in order to deal with hypoxia stress. Hypoxia-inducible factors (HIFs) drive the core transcriptional response to hypoxia in mammals. The HIF1α subunits are highly sensitive to various stimuli, including factors-inhibiting of HIF1 (FIH), sirtuins, and metabolites, while being controlled by prolyl hydroxylase domain enzymes (PHDs). These transcription factors are involved in maintaining normal tissue homeostasis and have a role in disease development, and recovery [160].

The mechanism of hypoxia response has been provided [19–21], where the key factor, HIFs, are the master regulators of oxygen-sensitive gene expression. Each HIF is a heterodimeric transcription factor complex which consists of one of the three different members-HIF1α, HIF2α, HIF3α, and the HIF1β is named as a standard ARNT subunit. The promoter of the hypoxia-regulated genes is known as hypoxia response element (HRE), which governs the levels of genes expression [161, 162]. The level of HIFα and HIFβ proteins, as well as HIF1 DNA-binding activity, improved exponentially with decreasing cellular oxygen tension in HeLa cells, with maximum levels at 0.5% oxygen and a half-maximum at 1.5% to 2% oxygen. HIF1α and HIFβ mRNA were found in all human, mouse, and rat tissues, and mRNA expression was moderately elevated in hypoxia-exposed animals. When mice were treated for anaemia or hypoxia, HIF1α protein levels were increased in vivo [163].

2.2.2 Hypoxia-Inducible Factor and its Subunits

Hypoxia is a situation in which the supply of oxygen to the body's tissues is inadequate. If there is a mismatch between the oxygen entering a cell and the oxygen demand of the same cell, a hypoxic condition can occur. Physiological and pathological events in a living cell cause an imbalance between oxygen supply and energy demand [164].

Hypoxia-inducible factor 1 (HIF1) is a transcriptional factor that plays an essential role in the adaptive response of cells to reduced oxygen tension. HIF1 regulates the expression of hundreds of genes in every particular cell type [165]. Because the target genome differs widely between cell types, the HIF1 transcriptome is expected to contain thousands of genes. HIF1, in particular, regulates developmental and physiological processes that either provide oxygen to cells or allow them to survive when they are deprived of it [166]. Under hypoxic conditions, HIF1 ultimately acts as a master regulator of multiple hypoxia-inducible genes.

At the molecular level, HIF is a heterodimer made up of subunits α and β, which are both helix-loop-helix transcription factors. HIF1α, HIF2α, and HIF3α are the three subunits that comprise HIFα; HIF1β, HIF2β, and HIF3β are the three subunits of the HIFβ subunit. Under normoxic circumstances, the subunits of the heterodimeric transcription factors HIF1α, HIF2α, and HIF3α are unstable, but the HIF1α subunit stabilizes under hypoxia and controls the expression of target genes. Cellular hypoxia induces α-subunit expression, which is then maintained at low levels in most cells with standard oxygen tension [167]. HIF1α levels are a crucial factor in HIF1 DNA binding and transcriptional activity [168]. HIF2α is structurally and functionally identical to HIF1; however, it has a more restricted tissue-specific expression and may be variably controlled by nuclear translocation. HIF2α is a protein with 48% amino acid identical to HIF1α that dimerizes with HIF1β and binds to the same target DNA sequence as the HIF1α·HIF1β heterodimer. It is likewise controlled by prolyl hydroxylation [169]. HIF2α is similarly connected to HIF1α and can interact with hypoxia response elements to increase transcriptional activity [170]. In addition, HIF2α, which is known to activate Epo expression, is accumulated in the liver. HIF3α (also known as IPAS), on the other hand, is implicated in the downregulation of the hypoxia response by an alternatively spliced transcription factor that may operate as an HIF1α inhibitor [171]. In the HLH and PAS structural domains, HIF3α is similar to

HIF1α and HIF2α, but it lacks a hypoxia-inducible domain [172]. HIF1α and HIF2α have been examined more thoroughly, while HIF3α and other HIF isoforms have received less attention [157].

HIFs trigger the transcription of an increasing number of genes involved in oxygen maintenance when they heterodimerize with constitutively expressed subunits. A growing number of genes important in maintaining cellular oxygen homeostasis are being transcribed [158]. It is suggested that various post-translational modifications, including hydroxylation, acetylation, and phosphorylation, influence the stability and function of HIF1.

Through the hydroxylation of proline and asparagine residues, oxygen controls the stability and activity of HIF1. Under normoxia conditions, two proline residues in the human HIF1α ODD domain are hydroxylated by prolyl hydroxylase domain proteins (PHDs). First, the von Hippel-Lindau protein (VHL) requires hydroxylation for the HIF-1 protein to bind to it. Then, a poly-ubiquitin chain is added to HIF-1 by an E3 ubiquitin ligase, which targets the protein for destruction by the proteasome. Additionally, O_2-dependent hydroxylation of asparagine residue suppresses the associate of HIF1α with the coactivators P300 and CBP (CREB binding protein), which inhibits the HIF1α transactivation function under normoxic circumstances shown by Liu *et al.* [173] in Figure 2.6. Under hypoxic conditions, on the other hand, the HIF1 subunit stabilizes and interacts with cofactors such as p300/CBP to modulate its transcriptional activity [174] displayed in Figure 2.7.

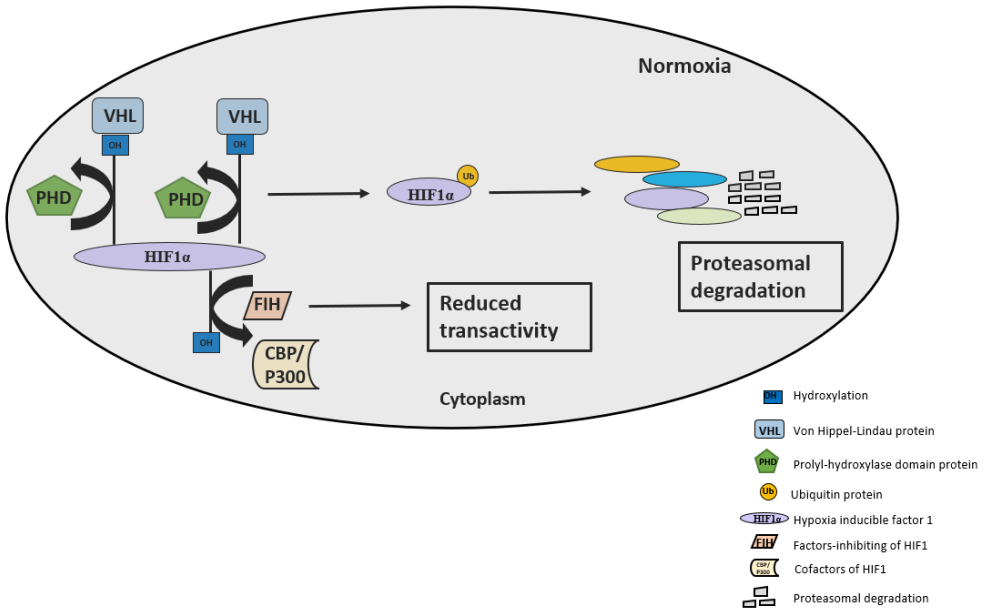

Figure 2.6. The process of HIF1α interact with p300/CBP cofactor under normoxia condition. Adapted from [173]

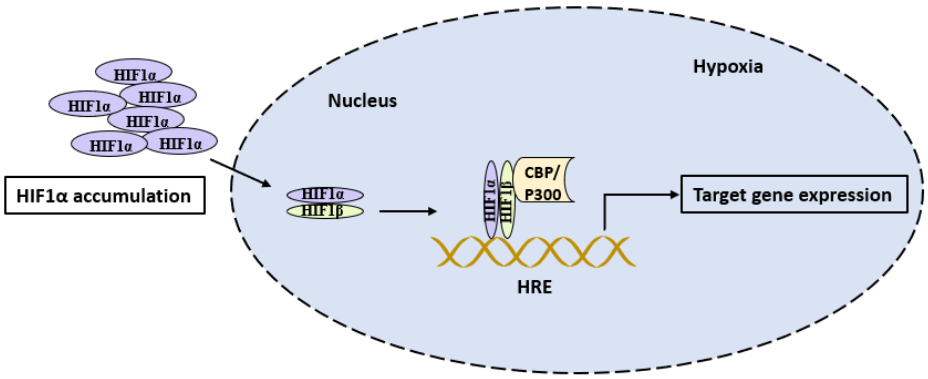

Figure 2.7. The process of HIF1α under hypoxia condition. Adapted from [173]

Target genes of HIF1 are involved in cell proliferation and survival, angiogenesis, and glucose metabolism. Furthermore, it has been found that HIF1 activation is associated with a range of cancers and carcinogenic pathways. As a result, inhibiting tumour development by blocking HIF1 or HIF-interacting proteins is possible. HIF1 might be a prominent target for anti-cancer therapeutics based on these findings [175].

2.2.3 The Prolyl-hydroxylase Domain (PHD) Proteins

Prolyl hydroxylase domain (PHD) proteins hydroxylate hypoxia-inducible factor (HIF)-α subunits in response to oxygen, signalling poly-ubiquitination and proteasomal degradation, and play a key role in controlling HIF abundance and oxygen homeostasis. PHDs are oxygen sensors that regulate the stability of the hypoxia-inducible factors (HIFs) in an oxygen-dependent manner. Although oxygen content is a primary determinant of the effectiveness of PHD-catalyzed hydroxylation processes, numerous other external and intracellular variables also have a substantial impact. PHDs may potentially use hydroxylase-independent ways to influence HIF activity. PHD2 and PHD3 are sensitive to feedback upregulation by HIFs, which is interesting because PHDs regulate HIFα protein stability. Varied PHD forms could contribute differently to pathological pathways such as angiogenesis, erythropoiesis, cancer, as well as proliferation, differentiation, and survival of cells on a functional level [176]. Loss of PHDs' expression or function, as seen in mice missing distinct PHD isoforms, causes multi-faceted pathophysiological alterations due to the numerous involvement of PHDs in many different processes. PHDs selectivity for distinct HIFα subunits in vivo and variable functions of PHD isoforms in diverse biological systems will require additional research in the future.

In mammals, three HIF prolyl-hydroxylases (PHD1, PHD2 and PHD3) have been discovered and proven to hydroxylate HIFα subunits by biochemical and candidate research recently. PHDs regulate the HIFα subunits of the hypoxia signalling pathway. A recent study has proposed that PHD2 monomer deficiency, for example, normalizes tumour vasculature, lowering tumour cell infiltration and metastasis predominantly via regulating HIF2α [177].

PHD1-3 are virtually universally expressed in tissues; PHD2 is typically the most abundant isoform, except for the testis, where PHD1 is the most highly expressed protein, while PHD3 expression plays a significant role in the heart [178]. Because of reprogramming towards anaerobic metabolism, PHD1-deficient animals have enhanced hypoxia tolerance in skeletal muscle, and liver [179, 180]. PHD2 deletion causes embryonic death owing to placental and cardiac problems, while postnatal loss leads to angiogenesis, polycythemia, and congestive heart failure [181]. Over 400 amino-acid residues are present in both intact PHD1 and PHD2 and share a conserved hydroxylase domain at their C-terminal.

Their N-terminal is much more distinct from C-terminal, but their functions are unclear. PHD3 deficiency leads to hyposympathetic nervous system dysfunction and a drop in blood pressure [182]. Although three HIF prolyl hydroxylases, PHD1, PHD2, and PHD3, have been discovered as possible catalyzers of this process, the role of each isoform in the physiological control of HIF is still unknown [183].

PHD2, a short interfering RNA, has been demonstrated to be adequate in all human cells studied for stabilizing and activating HIF1α under normoxia. However, PHD1 and PHD3 exhibit no influence on the stability of HIF1α in normoxia or when cells are re-oxygenated after being subjected to hypoxia for a short period. In vivo, therefore, PHDs serve a variety of roles, with PHD2 serving as the primary oxygen sensor that maintains low HIF1α steady-state levels in normoxia [184]. Impressively, PHD2 is elevated in response to hypoxia, implying that the oxygen tension drives an HIF-dependent auto-regulatory mechanism. PHD2 is firstly found to be the most significant hydroxylase in a wide range of cultured cells. However, further investigation indicated that one of the reasons for this is because it is expressed more often than other isoforms [183, 184]. The relevance of PHD1 and PHD3 isoforms is identified when their expression levels are boosted via transfection, particular in the hydroxylation of HIF2α.

There is no doubt that hypoxia and HIF influence a range of PHD activities. Although there has been no evidence of an alternate enzymatic activity for these oxygen sensors, the argument about hydroxylase-independent functions of PHDs is still going on. On the other hand, Steric hindrance has been proposed as a method by which PHDs govern their targets. Furthermore, recent research has discovered that iron chaperones are required for PHD activity, implying that all HIF-regulating enzymes (PHDs, VHL) are recruited and maintained in a large complex to improve enzyme performance [185]. Moreover, evidence is presented that PHD activities are not limited to the HIF pathway and have a broad physiological platform (including neural signalling). Because the HIF-independent actions of PHDs have been proven to be confined to a single isoform, the demand for isoform-specific PHD inhibitors to prevent adverse effects in a specific cell type or pathological condition is becoming increasingly significant.

2.2.4 The von Hippel-Lindau Protein (VHL)

Under normoxic circumstances, the product of the von Hippel-Lindau (VHL) tumour suppressor gene facilitates ubiquitination and proteasomal degradation of HIF1α through interaction with the core of the oxygen-dependent degradation domain of HIF1α. In the crystal structure of VHL, the area where VHL contacts with HIF1α coincides with a potential macromolecular binding site. This VHL pattern is also a target for mutations, which affects the connection with HIF1α and leads to degradation. Hypoxia-induced nuclear translocation of HIF1α and hypoxia-dependent signalling in the nucleus are part of a multistep system that protects HIF1α from degradation by VHL. During this procedure, VHL is not liberated from HIF1 [186]. Two recent investigations have revealed unique properties for VHL in the regulation of HIF1α function: activation of a natural HIF1α antisense transcript in VHL-deficient cells, leading HIF1α function to be negatively regulated. Physical interaction of VHL with HIF1α, on the other hand, has recently been addressed, suggesting that it may be used to target HIF1α for protein degradation [187]. Studies have demonstrated that VHL interacts with HIF1α to induce ubiquitin-proteasomal degradation of HIF1α in normoxic circumstances. One of the hotspots of cancer-causing mutations in the VHL gene is the HIF1α binding domain of VHL [188]. Indeed, a common tumour mutation in this area of VHL prevented the formation of the VHL-HIF1α complex, suggesting that the VHL-HIF1α interaction is critical for the tumour suppressor activity of VHL.

HIF1α protein degradation is controlled by O_2-dependent prolyl hydroxylation, which allows E3 ubiquitin-protein ligases to ubiquitylate the protein. The von Hippel-Lindau tumour-suppressor protein (VHL) found in these ligases binds selectively to hydroxylated HIF1, while the proteasome quickly degrades ubiquitinated HIF1 [175]. In addition, the inactivation of the VHL protein causes VHL diseases, which is characterized by the development of numerous vascular tumours.

2.2.5 Mathematical Modelling of Hypoxia Response

By proposing biologically plausible mechanisms and establishing empirically testable predictions, systems-level models of the HIF pathway will give a dynamic and mechanistic knowledge of how physical and chemical processes combine to create complex

cellular responses to hypoxia [189]. Therefore, several mathematical models for HIF signalling have been presented to achieve the goal. The following models have confirmed this hypothesis and offered mathematical explanations for the process that causes such switching behaviour [23][26]. Due to limitations in the availability of experimental data, these models are primarily based on steady-state experimental data and do not incorporate the dynamics of the hypoxia response. Furthermore, early models exclusively include PHD as the oxygen sensor in the HIF network, leaving the control of HIF transcriptional activity unexplored. FIH is involved in more recent models, although it has been used to investigate other aspects of the network, such as the regulation of particular groups of HIF-dependent genes [190].

Over the last two decades, a large number of empirical studies has focused on utilizing mathematical modelling to illuminate both general theories and quantitative explanations behind molecular networks, with the aim of enabling to accurately predict and model significant networks of the cell [191]. The first theoretical model of the HIF network, which led to the assumption that HIF activity responds to diminishing oxygen gradients in a sharp switch-like manner, was developed in [19].

The HIF1α signalling network was studied by using a combination of mathematical and experimental methods in [25]. It is accomplished by developing an iterative dynamic model that is verified using experimental data and has adequate predictive ability to properly represent the spatial and temporal response of HIF transcription to hypoxia. Nguyen *et al.* [25], provided a dynamic, ordinary differential equation-based model from the major components of the verified and published HIF1α network to give a quantitative framework for understanding the HIF pathway. Due to the limited availability of experimental data, existing models have under-represented this critical stage.

Since Kohn *et al.* [19] established mathematical modelling of the HIF pathway, as well as the construction of molecular interaction maps (MIMs), various HIF models have been created primarily concentrating on four topics; (1) HIF nuclear accumulation and transcriptional activity have a switch-like behaviour that is mediated by oxygen; (2) Molecular micro-environment sensitivity of HIF; (3) Function of FIH in HIF activity and stability response modulation; and (4) Temporal dynamics of the HIF response to hypoxia.

The existing HIF models are mainly based on biological reaction kinetics and use ordinary differential equations (ODEs), which is an effective technique for dealing with dynamic

network behaviour. For each component of the network interaction diagram, ODEs were developed using mass action or Michaelis-Menten laws. For some processes mediated by PHD and the interaction between VHL and proline hydroxylation HIF proteins, model reductions were achieved utilising quasi-steady analysis in some models.

In [19], Kohn *et al.* proposed that a small subsystem may control the fundamental elements of the cellular response to hypoxia, which has a stinging oxygen-dependent shift. As a result, a network molecular interaction map (MIM) was presented, and a conserved core subsystem was identified that could govern the activity of hypoxia response promoter elements based on oxygen concentration. Hypoxia-inducible transcription factor (HIFα·HIFβ heterodimer), proline hydroxylase domain (PHD), and the von Hippel-Lindau (VHL) protein are all part of this primary subsystem. Kohn *et al.* have proposed a hypoxia-responsive promoter element-dependent response mechanism in hypoxia and growth factor signalling. Their investigations uncovered the mechanisms behind these remarkable transitions. The model in [19] demonstrates how to code subsystems for a computer-assisted parameter space inquiry, how to clarify switch-like activity, and how this behaviour predicts response characteristics, allowing for solid connections between theoretical experiments.

By computationally incorporating a scheme of ordinary differential equations (ODEs) comprising various of molecular species relevant to hypoxia, the switch-like response elements of HRE occupancy have been proposed by Kohn *et al.* [19]. The smaller components responsible for the switch-like response are not identified by the huge system, which may be found in other networks. In addition, numerical solutions do not provide the same level of understanding as mathematical formulations. Simultaneously, solving the sort of nonlinear differential equations that represent the reactions conceptually is nearly impossible. Methods are needed that both reduce the network to its essential components and do not rely only on statistical solutions in this scenario.

Except for the model in [19], a different related method is reported. The model in [23] described the observed behaviour with a thorough quantitative analysis. The model is accomplished by connecting essential pathway identification via extreme pathway analysis (EPA), which is entirely based on network topology, with detailed numerical analysis of the controlling differential equations in the model, in which the space of mostly the unrevealed rate constants from the differential equations are enabled to explore. Furthermore, Yu *et al.* [23] suggested that the HIFα pathway of excessive synthesis and degradation is

the driving force behind the rapid response. In particular, the flux redistribution among the elementary routes as a function of oxygen concentration is studied in detail. They also identify the essential molecular species engaged in the network subcomponent and demonstrate quantitatively how the response of this subcomponent is identical to the overall response and hence is responsible for it.

Despite both models of Kohn *et al.* [19] and Yu *et al.* [23] depicting a steady state in the HIF response at extremely low oxygen tensions, the experimental data they employed show that the HIF response is diminished at near-hypoxic oxygen levels. Kooner and colleagues [192] expanded Kohn's model to include the function of oxygen in mediating HIFα with PHD, HIFβ and HRE reactions, as well as its nuclear export to account for the inconsistency. In comparison to Kohn's model, in [192], the model proposed by Kooner *et al.* assumes that HIFα and PHD interact in an oxygen-dependent way, while the dissociation process is oxygen-independent. Additionally, HIF1α·HRE and HIF1α·HIF1β dissociations and nuclear export of HIFα were believed to be linear in relation to oxygen concentration. They were using data on HIF-export from the nucleus and its cytoplasm degradation following re-oxygenation. They also suggested that hydroxylation and ubiquitination can occur both in the nucleus and cytosol and indicated that the negative feedback by PHD can be ignored.

In summary, the models produced so far have mainly explored the association between oxygen levels, a small number of microenvironmental variables, and the HIF response (protein stability and transcriptional activity) utilising the core components of the HIF pathway (HIF, VHL, PHD and HRE). Whereas the primary components may characterise the HIF response, the models were built on the basis of many assumptions. These assumptions are sometimes used to simplify the modelling process, but they are more commonly made due to a lack of experimental evidence. Nevertheless, most HIF models to date have been built using currently accessible third-party data, however, specific models, such as [25][193], have benefited from an iterative process of experimental and mathematical validations. Furthermore, except for the most recent models, the scarcity of dynamic experimental data has forced most models to concentrate on steady-state behaviour.

2.2.6 The Implication of HIF1 in Diseases Development

Hypoxia and the HIF pathway have been connected to embryonic development and the pathophysiology of a variety of diseases in humans. HIF1 is a transcriptional activator that is activated by oxygen and is involved in tumour angiogenesis, and mammalian development [174]. The prominence of HIF1 as a transcription factor and the wide range of activities it influences imply that it might have significant therapeutic consequences. The different factors of HIF1 regulation provide a wide range of treatment options [157]. It suggested that HIF1 overexpression has been identified in a number of malignancies, and medication that targets HIF1 might be a potential cancer treatment strategy. HIF1 appears to have a vital role in the prevention of myocardial and cerebral ischaemia, as well as the development of pulmonary hypertension and cancer, according to new research. HIF1 activity modification by genetic or pharmacological techniques might give a novel treatment strategy to these frequent causes of death [194]. In addition to ischaemic stroke, emerging evidence indicates that HIF1 activity and the expression of downstream genes, including vascular endothelial growth factor and erythropoietin, are changed in some neurodegenerative diseases [195].

One of the main unaddressed health concerns of the world is neurodegenerative diseases, which are defined by a gradual worsening in the structure and function of the brain. As a result, it is critical to identify therapeutic targets in order to develop successful treatment approaches for these diseases [196]. Recently, emerging evidence has indicated that inducing the hypoxia signalling pathway while simultaneously stabilizing and activating the transcription factor HIF1 might mediate neuroprotective effects. HIF1 has been demonstrated to regulate the expression of genes encoding in oxygen transport, glycolytic metabolism, angiogenesis, cell survival, apoptosis, and other cell-survival activities [48][196]. The HIF1 signalling pathway is engaged in various physiological responses, with HIF activity being linked to the control of numerous metabolic pathways. Consequently, activating the HIF1 signal transduction system and increasing the expression of its protective target genes might be an effective neuroprotective technique for treating or preventing neurodegenerative diseases [196].

In the biology of breast cancer and prostate cancer tumours, HIF1α has emerged as a key transcription factor [197]. Increased HIF1 activity may improve survival during hypoxia

and enhance angiogenesis at vascular disruption or malfunction locations. Reduced HIF1 activity might prevent pathogenic tissues with hypoxic areas, such as solid tumours, from surviving or angiogenic activity. Because of its critical function in the activation and infiltration of macrophages and neutrophils into afflicted tissues, evidence has recently been collected that inhibiting HIF1 activity may also help to avoid inflammation [198].
Based on the current understanding of the complex regulation of this protein, targeting HIF1α to kill or suppress hypoxic cancer cells has explicitly now become possible. Farnesyl transferase inhibitors, which are now in clinical trials, might work in part by preventing HIF1α. HIF1 inhibition should have a wide range of implications. Angiogenesis is required for the growth of a multicellular tumour. By reducing the expression of pro-angiogenic genes, inhibiting HIF1α will slow tumour growth.
HIF1α expression appears to be linked to cancer growth and development in most human malignancies. Hypoxic cancer cells are also more resistant to radiation and chemotherapy, while HIF1, surprisingly, works to prevent resistance to both. As a result, inhibiting HIF activity might improve the effectiveness of current radiation and chemotherapy treatments. It might slow down tumour development and spread by lowering the effects of angiogenesis. Regardless of tumour grade, stage, or other indicators, overexpression of HIF1α is related to radiation resistance and higher mortality in head and neck cancer [199, 200].

2.2.7 Summary

The fate of cells under extreme stress, such as heat shock and hypoxia, is one of the most critical topics in cell biology. Therefore, it is vital to understand how organisms respond to extreme environmental stresses and/or pathological situations to create basic metabolic therapy techniques to enhance their survival in these circumstances.
One of these stressors, HS, which may be brought on by a temperature rise of only a few degrees (°C), poses a serious threat to life [3–5]. It is now widely established that cells engage signalling pathways that result in the transient expression of heat shock proteins (HSPs) in response to HS [7]. In addition, various genetic and physiological factors result in the rapid activation of HSPs in response to stress, which is known as the heat shock response (HSR). Compared with experimental studies of HSR, there has been some progress in its mathematical modelling [4][16, 17]. In order to understand the structure of

the parameter space, a Monte Carlo (MC) scan of the parameter space was carried out by Magin *et al.* in their proposal of a data-driven dynamical model of the heat shock response in *Chlamydomonas reinhardtii* in [16]. Peper *et al.* [15] model includes interactions between mRNA molecules and HSP70, heat-induced misfolded proteins, and dimeric and trimeric forms of HSFs. However, the lack of consideration for the specifics of HSPs-regulated transcription is a drawback of this model. The heat shock response of eukaryotes related with HSPs, HSFs and HSE, as well as HSP-mRNA molecules, stimulation signals, and kinases, was examined using the model in Rieger *et al.* [17].

Hypoxia (low oxygen tension), another external stressor, causes various metabolic issues that have immediate and long-term effects on cell physiology. Since the discovery of hypoxia-inducible factors (HIFs), much progress has been made in our knowledge of how hypoxia induces the expression of genes that are controlled by oxygen [158]. Systems-level models of the HIF pathway will provide a dynamic and mechanistic understanding of how physical and chemical processes interact to form complex cellular responses to hypoxia by putting forward biologically plausible mechanisms and making experimentally tested predictions [189]. Therefore, several of mathematical models for HIF signalling have been provided to accomplish the aim. In [19], the first mathematical model of the HIF network was created, which established the presumption that HIF activity reacts to decreasing oxygen gradients in quick switch-like manner. The model in [23] provided a complete quantitative analysis to characterise the observed behaviour. The model is achieved by tying crucial route identification to extreme pathway analysis (EPA). The concept put out by Kooner *et al.* [192] differs from Kohn's model in that it posits that the dissociation process is oxygen-independent, whereas HIF and PHD interact oxygen-dependently. Utilizing the essential elements of the HIF pathway, the models developed so far have primarily investigated the relationship between oxygen levels, a select few microenvironmental factors, and the HIF response (protein stability and transcriptional activity).

Overall, several different correlation models have been mentioned above, and these models are based on the heat shock and hypoxia response. Although there are some drawbacks to each model, their establishment and development also laid the foundation for the development and construction of future frameworks.

Chapter 3

The Role of SUMOylation in Cellular Stresses Response

3.1 Introduction

SUMOylation is an essential process of protein expression that is related to varying cellular activities. It is generally agreed that cellular stress causes global increases in protein SUMOylation. SUMO conjugation levels in cells are affected by numerous factors, including cell cycle stage [201], differentiation [202] and a variety of stresses, such as heat shock [36][203], hypoxia stress, oxidative stress[204–207], DNA damage [208] and viral infection [209], according to early research on mammalian and yeast cells. Each of these factors affects the level of transcription or activity of proteins involved in SUMOylation. Exposure to environmental or metabolic stress directly alters the equilibrium between SUMOylation and deSUMOylation for many substrate proteins, which has become clear recently. Changes in ambient temperature, osmotic pressures, oxygen tension or oxidative state, and ischemia insult have all been shown to cause a significant rise in global SUMO-2/3 conjugation in a variety of cell types, indicating that this is most likely to be a cellular defensive response.

However, when exposed to heat shock stress, SUMO2/3 conjugation increases significantly, while SUMO1 conjugation seems unaffected. Likewise, oxidative, ethanol and osmotic stresses all caused a rapid rise in global SUMO2/3 conjugation [204]. In several physiological systems, including oxygen/glucose deprivation and hypothermia in neurons,

stress-related increases in SUMO conjugation have been described [40]. Increased SUMO conjugation due to stress is a frequent physiological response, suggesting that protein SUMOylation might be a protective reaction. Although cellular stress-induced increases in SUMOylation seem to be a common occurrence, it is vital to note that specific substrates are changed differently. For instance, while there is a global rise in SUMOylation after heat shock, the SUMOylation level of some substrates remains constant. In contrast, that of others reduces, demonstrating that substrate SUMOylation under these conditions is a controlled specific stress response rather than a nonspecific increase [36].

Hundreds of SUMOylation substrates have been described since the discovery of SUMOylation as a modification of the nuclear pore component RanGAP, and many more have been found through proteomic investigations [210]. SUMOylation is well known for its function in genome integrity, nuclear structure, and transcription in nuclear proteins. However, it has also been shown that SUMOylation is involved in signal transduction, trafficking, and modification of cytosolic and integral membrane proteins outside of the nucleus [211]. Since many of the reported SUMO target proteins are recognised oncogenes and tumour suppressors, overexpression of the SUMO system leads to enhanced cell proliferation and invasion and decreased apoptosis in malignancies. These findings will prompt more research into the regulatory processes behind the SUMO machinery's dynamic distribution and activity.

Moreover, the involvement of protein SUMOylation in health and disease is a relatively recent subject of study. SUMOylation has been associated with cancer, diabetes, cardiovascular disease [212], and clinically significant neurodegenerative diseases, including dementias such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) [34]. The impact of SUMOylation on crucial cell cycle regulators will give further functional insight into the role of SUMOylation in cancer. A functioning SUMO pathway appears to be required for certain cancers. Interfering with SUMO signalling has been found to inhibit tumour development. It needs to be seen if SUMOylation is essential for tumour development more generally. A more comprehensive study of the expression levels of several elements of the SUMO machinery and global SUMOylation levels across a diverse group of malignancies is required. In addition, the SUMOylation levels of specific target proteins in these malignancies will be intriguing to investigate. This information might be used to identify cancer types that potentially benefit from SUMOyla-

tion machinery inhibition. To reduce risk in healthy organs, another challenge will be to interfere with SUMOylation in cancer cells selectively. A functional SUMOylation system may also be required for rapidly developing cells in the gut, bone marrow, and elsewhere [213]. Nevertheless, future research into whether interfering with SUMOylation could be a viable anticancer therapy would be fascinating.

In conclusion, global SUMOylation is an essential predictor of cell fate, although the existing data for many individual proteins are unclear and occasionally conflicting. As a result, much more study is needed to better understand the complexity of deSUMOylation enzyme regulation, target, and effects, despite the significant advances.

3.2 The Mechanism of Reversible SUMOylation

3.2.1 The SUMO Family and Functions

SUMOs are one of several proteins similar to ubiquitin (Ub) that were discovered in the 1990s [214]. SUMOs are a class of proteins that are attached to target proteins covalently and reversibly [215]. SUMO has 97 amino acid residues, sharing a similar molecular mass ($\sim$10kD), three dimensional (3-D) structure and a C-terminal di-glycine motif with the Ub [216–218]. However, it is worth noting that SUMO and Ub differ significantly: there is only $\sim$18% amino acid identify shared between the two protein types and the surface charge distribution on SUMO is different, in terms of obvious positive and negative regions from Ub [216][219]. Geiss-Friedlander [219] mentioned that in-depth investigations on SUMO substrates have led to the discoveries of substantial numbers of target proteins that appear to be involved in most of the essential cellular functions.

The SUMO proteins are widely expressed in all eukaryotes. The yeast, *Caenorhabditis elegans*, and *Drosophila melanogaster* are identified as single SUMO proteins, known as Smt3 and Pmt3, respectively. Plants and vertebrates all contain various SUMO proteins. There are four SUMO paralogues in a mammal, called SUMO1, SUMO2, SUMO3, and SUMO4 [31]. It has been shown that SUMO1$\sim$SUMO3 could be globally expressed, whereas the expression of SUMO4 is exclusively found in the kidney and spleen [220]. All SUMO proteins are produced immaturely with a variable-length C-terminal stretch that designates the mature protein's C-terminal. SUMO-specific proteases must delete

the C-terminal extension to conjugate SUMO to targets. A common characteristic of ubiquitin-like modifiers appears to be the expression of peptide modifiers as precursors [219]. In addition, in [204], Saithon and Hinchey proposed that plants might even contain up to eight paralogues. Before conjugation, all SUMO isoforms are expressed as precursor proteins that need carboxy-terminal proteolytic process. The mature forms of SUMO2 and SUMO3 $\sim$95% identify with each other, while each has $\sim$45% identify with SUMO1. In most cases, SUMO2 and SUMO3 are indistinguishable from each other due to their highly consistent identification, and therefore these two are often referred to as SUMO2/3. Furthermore, there is approximately a 45% sequence similarity between mammalian SUMO paralogues and Smt3 in lower eukaryotes. SUMO1 and SUMO2/3 have diverse roles because they are attached to various target proteins in vivo [204][221, 222]. However, the function of SUMO4 is unknown, as its ability to convert to the mature conjugated form in vivo cannot be determined [220][223].

SUMO1, also known as UBL1 [224], PIC1 [225], sentrin [226], GMP1 [227] and Smt3c [228], is an 11kDa protein that has been identified as a binding partner of the RAD51/52 nucleoprotein filament proteins, which facilitate DNA strand exchange, and PML (promyelocytic leukaemia), a component of multiprotein nuclear complexes, by different groups separately [224][226, 227]. SUMO1 is a highly conserved, small ubiquitin-related modifier that has been found covalently attached to a number of biological proteins [227][229–231]. SUMOylation of the nuclear pore protein RanGAP1 was described in the earliest findings that SUMO1 operates, as a covalent protein modification (Ran-GTPase activating protein 1) [227][231].

The carboxyl terminus of SUMO1 and a lysine side chain of the target protein is thought to create an isopeptide bond, similar to ubiquitin [229, 230][232]. The binding of SUMO1 to cellular proteins has been considered to have a role in various essential physiological functions, including nuclear transport, cell cycle regulation, tumourigenesis, inflammation, and viral infection response [233, 234]. SUMO1 conjugation has been suggested to have antagonistic effects with ubiquitin conjugation and/or to control the association of target proteins with other cellular components [232][235].

It has been reported that SUMO2 (also known as sentrin 2 [236], Smt3b [228] and GMP-related protein [227]), and SUMO3 (known as sentrin 3 [236] and Smt3a [228]), and the proteins were then isolated and demonstrated to conjugate substrate proteins [236, 237].

DNA sequence analysis has also suggested a SUMO4 isoform, predicting a 95-residue protein with 87% amino acid similarity to SUMO2. In high cellular stress, externally produced mature forms of SUMO4 have been observed to be conjugated [238]. However, unlike the other SUMO genes, the SUMO4 gene does not include introns, suggesting that it might be a pseudogene [239]. Furthermore, despite the presence of SUMO4 mRNA in the kidney, lymph nodes, and spleen [220][239], no endogenous SUMO4 protein has been found. Finally, SUMO4 has a crucial proline residue, making it uncertain if the precursor SUMO4 protein might be matured to conjugate to target proteins, even if it is produced endogenously [223].

3.2.2 SUMO-Specific Enzymes

Ubiquitin is a common protein that binds to other proteins via a peptide bond formed by C-terminal glycine on the substrate, discovered in the 1970s. Four enzymes are involved in SUMO conjugation and deconjugation, including a ubiquitin-like (Ubl) activating enzyme (E1), a ubiquitin-conjugating enzyme (E2; also known as Ubc9), and a ligase enzyme (E3) for conjugating SUMO to a target protein, and a sentrin (another name for SUMO)-specific protease (SENP) for deconjugating SUMO from the target protein. SENPs are involved in processing precursor forms of SUMO and in removing SUMO or SUMO chains from modified proteins [37].

• **The SUMO-Activating Enzyme E1**

The SUMO E1 enzyme was detected in *S. cerevisiae*, because of its sequence similarities to the ubiquitin E1 enzyme (Uba1) [229]. SUMO Activating Enzyme (SAE; E1) is a heterodimer including SAE1 and SAE2 subunits, which are known as Aos1 and Uba2 in yeast [240–242]. In order to initiate the SUMO modification process, Aos1 and Uba2 will firstly activate mature SUMO in an ATP-dependent manner [219]. It has been proposed that the human homologs of yeast *S. cerevisiae* Aos1 and Uba2 are Sua1 and hUba2, respectively. SUMOylation processing is an ATP-dependent reaction, and E1 is an essential component in the multistep pathway [241][243]. In the activation step, which is after the mature SUMO step, the E1 enzyme heterodimer Aos1 and Uba2 utilise ATP to adenylate the C-terminal glycine of mature SUMO and release free AMP, and then the formation of a high-energy thioester bond between the mature SUMO C-terminal and E1 enzyme

[240, 241][244]. After that, thioester bonds can connect mature SUMO and the cysteine residue of E1. As a result, SUMO E1 is produced eventually, which prepares for the E2 conjugating step.

The SUMO E1 enzyme, unlike Uba1, is a heterodimer made up of the Aos1 and Uba2 subunits (also referred to as Sae1 and Sae2 in mammals). According to structural research, an adenylation domain, a catalytic domain, and a Ubl domain are three domains of Uba2 having structural similarities to ubiquitin and other Ubl modifiers. A C-terminal extension containing a predicted nuclear localisation signal (NLS) and two SUMO-interacting motifs (SIMs) can be found in the mammalian Uba2 subunit. While this area is not required for E1 function in vitro or in *S. cerevisiae* in vivo, it is most likely to be involved in mammalian cell regulation functions [245, 246].

Similarly to the E1 for Ub, the SUMO-activating enzyme E1 catalyses a three-part process. Initially, SUMO's C-terminal carboxyl group attacks ATP, resulting in the formation of a SUMO C-terminal adenylate and the release of pyrophosphate. The active site cysteine of E1 then assaults the SUMO adenylate, releasing AMP and establishing a high-energy thioester bond between the E1 and SUMO's C-terminus. Subsequently, the active SUMO in the E2 is transported to a cysteine. The crystal structure of the corresponding E1 for the Ubl Nedd-8 reveals that each of the stages is catalysed by three different domains [247]. A single SUMO-activating enzyme is present in most species, and it is necessary for the conjugation of all SUMO variants to all substrates. Surprisingly, the SUMO E1 is a heterodimer, while the Ub E1 is a monomer; however, both SUMO enzyme components are connected to the Ub enzyme [248].

As predicted, the regulation of E1 activity causes broad alterations in the highly dynamic SUMO proteome. Overall, E1 regulatory mechanisms respond rapidly in various systems that are affected by environmental changes. Most cellular substrates are deSUMOylated as a result of both enzymes being temporarily inactivated [249, 250]. Post-translational modifications (PTMs) have also been demonstrated to regulate E1 activity. The SUMOylation of Lys 236 on the human Uba2 subunit does not affect SUMO adenylation or E1~SUMO thioester production, but it does affect the subunit's interaction with the E2 enzyme. Under heat shock conditions, Uba2 SUMOylation is consistently decreased, which associates with enhanced global SUMOylation [251].

• **The SUMO-Conjugating Enzyme E2**

Ubc9 is the single E2 enzyme involved in SUMO conjugation. It generates a SUMO~Ubc9 thioester bond by interacting with the E1 to take SUMO. The charged E2 then interacts with the substrate, generally an E3, and SUMO is transferred to the substrate consequently. Therefore, the E2 enzyme has binding interfaces for the E1, the substrate, the E3 and also for SUMO [252]. All Ubc9 close relatives have the same Ubc structure, which is quite similar to other E2 ubiquitin pathway enzymes [253, 254]. Since Ubc9 is the only identified SUMO E2, and because it plays such an essential role in SUMOylation, it is expected that regulating its catalytic performance has broad cellular SUMOylation impacts equivalent to those of the E1. Nevertheless, regulation of particular substrates, or E3 binding interfaces, is predicted to focus on only a subset of substrates.

The SUMO conjugating enzyme Ubc9 plays an essential role in SUMOylation because it offers target selection through interactions with its various surfaces. Ubc9 proteins are SUMOylated in both humans and *S. cerevisiae* [205][255–257]. SUMOylation of mammalian protein Ubc9 has no effect on Ubc9-SUMO thioester synthesis; however, it does influence target protein selectivity [258]. E2 functions are generally regulated by regulators of their expression level or catalytic activity. In contrast to general Ubc9 function regulators, E2 modifications that interfere with specific substrate interactions, such as acetylation, N-terminal SUMOylation in mammals, and phosphorylation, show a more selective form of regulation, as these modifications only affect a subgroup of SUMO substrates [259–262].

As mentioned above, in the E1 enzyme activating step, a formation of a thioester bond with SUMO happens first, and then the SUMO proteins are transferred from E1 to E2 with the help of a single E2 SUMO-conjugating enzyme Ubc9. Ubc9 is unusual in E2 conjugating proteins because it plays a specific part through the direct interaction of sequence and structural motifs with its substrate. In other words, in this process, Ubc9 forms a thioester bond with not only SUMO but also non-covalent interaction with SUMO. Compared with other E2 ubiquitin-conjugating enzymes, there is a firm electrostatic dipole, where the N-terminal region with positive and hydrophobic residues is highly conserved in all Ubc9s. In general, Ubc9 plays an important role in biochemical processes involving SUMOylation, including protein degradation, DNA repair machinery, cell cycle progression and nuclear integrity. Based on these findings, a hypothesis has been proposed that Ubc9 plays a

significant role in cancer progression.

• **The SUMO-Ligating Enzyme E3**

The SUMO ligation enzyme (E3) has been documented in yeast, and mammals are implicated in the ligating step of SUMO modification. Generally, SUMO and target lysine residue produce an isopeptide bond when an E3 ligase binds to charged E2. In this step, the conjugation is between SUMO and target through SUMO E3 ligases. The protein-protein interaction domains of E3 bind to and identify targets, contributing to the ligation specificity.

Recently, three different types of SUMO ligases (E3) have been reported. One group consists of members of the PIAS (protein inhibitor of activated STAT) family [263], which were originally discovered as inhibitors of STAT transcription factors [264]; another group is composed of a domain in the large vertebrate nuclear pore protein RanBP2 [265], and the third group contains the polycomb group protein Pc2 [266]. These proteins match the description of an E3 because they bind the E2, bind the substrate, and enhance SUMO transfer from the E2 to the substrate in vitro [267]. These SUMO E3s, like the RING domain-containing E3 implicated in ubiquitylation, do not tend to exist by forming covalent intermediates with SUMO but rather by bringing Ubc9 and the substrate together. They may also turn on Ubc9. The presence of E3 in the SUMO pathway was previously questioned since SUMO is selective for the Lys residues that are actually changed in vivo [240][242]. However, the great majority of SUMOylation in yeast is E3-dependent, and E3 improved SUMO attachment to all in vitro substrates studied [265][268–270]. These findings suggest that E3 is involved in at least the majority of SUMOylation in cells.

E3 ligases catalyses the transfer of SUMO from E2-conjugating enzyme to the substrate. As a result, they interact with Ubc9~SUMO and the substrate to bring them together. Catalysts are recycled in the process, allowing a single enzyme to modify the substrate several times. Therefore, an E3 ligase's capacity to function at substoichiometric concentrations relative to its substrate is a distinguishing feature. Catalysts are typically not consumed during the process; however, all known SUMO and ubiquitin E3 ligases are substantially auto-modified. Consequently, auto-modification must be differentiated from substrate modification (trans-reaction). Therefore, characterising true SUMO E3 ligases needs a thorough biochemical investigation, preferably in combination with structural research, to establish substoichiometric trans-reactions and understand how the E3 releases SUMO

from the E2 for transfer to the substrate. Enhancement of substrate SUMOylation in cells does not always imply E3 ligase action since indirect effects such as regulatory co-factors or SUMO protease inhibition might potentially result in the same effect [252]. All SUMO E3 ligases appear to have a wide range of substrates and distinct targets. The global SUMO proteome is very dynamic and continuously changes, for example, throughout the cell cycle or in response to various stress stimuli, leading to stimulus-specific group SUMOylation and deSUMOylation [271–274].

• **The SUMO/Sentrin-Specific Protease SENP**

Ulps (Ubiquitin-like Proteases) in yeast and SENPs (SUMO/Sentrin-Specific Proteases) in humans belong to the cysteine proteases. The enzymes in the two protein families are essential for the SUMO deconjugation process. However, the significant difference between Ulps and SENPs is that the former exists in lower eukaryotes, while the latter can only be found in higher eukaryotes. Additionally, SENPs act as a trigger factor in converting pre-SUMO into a mature form [31]. In mammals, six SENPs are discovered, SENP1–3 and SENP5–7, and three groups will be used to divide these six SENPs [41]. SENP1 and SENP2 are members of the first group, which have specificity for deSUMOylating all three mammalian SUMO isoforms (SUMO-1, -2, -3) from target proteins. The second group consists of SENP3 and SENP5, favouring SUMO-2/3 over SUMO-1 as deSUMOylation substrates and are localised in the nucleus. SENP6 and SENP7 are the third group members, which have an additional loop inserted in the catalytic domain and appear to prefer SUMO-2/3 and associate with SUMO-2/3-chain editing.

SUMO proteases are enzymes that deconjugate SUMO from proteins in a direct manner. These enzymes break between the terminal Gly of SUMO and the substrate Lys with pinpoint precision [275]. Specific SUMO proteases are also involved in the maturation of SUMO precursors, which indirectly affects SUMO conjugation. Upl1 from *S. cerevisiae* was the first SUMO protease to be discovered (Ubl-specific protease 1). A second budding yeast SUMO protease (Ulp2) and suspected SUMO proteases in many other species were uncovered by comparing the sequence of the Ulp1 catalytic region to sequence databases [276–278]. The first human protein found to be comparable to Ulp1 was sentrin-specific protease 1, a SUMO-specific protease (SENP1; SUMO is also known as sentrin [279]). Additional potential human SENPs were recognised after more comprehensive database searches, and later research revealed that humans express six SUMO proteases of the

SENP class: SENP1-SENP3 and SENP5-SENP7. Furthermore, three main SUMO proteases have been discovered in humans: deSUMOylating isopeptidase 1 (DESI1), DESI2 and ubiquitin-specific protease-like 1 (USPL1), which have little in common with the Ulp/SENP (ubiquitin-like protease/sentrin-specific protease) [280]. However, as further experimental studies found, SENP8 does not interact on SUMO paralogs, but rather the ubiquitin family member Nedd8 [281, 282]. Thus, SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7 are the six SUMO-specific Ulp/SENP family members encoded in the human DNA. The Ulp1 branch is associated to SENP1, SENP2, SENP3 and SENP5, while Ulp2 branch is connected to SENP6 and SENP7, according to sequence analysis comparisons of human SENPs with lower eukaryotes Ulp1 and Ulp2 [31].

The SUMO system relies on controlled deconjugation of SUMO from its targets to ensure the flexibility of protein interaction networks. A class of cysteine proteases known as SUMO-specific proteases catalyses the deconjugation process. Furthermore, the mutation of the C-terminal of the SUMO precursor requires the action of certain family members as facilitators [31].

SENPs have been shown to function as enzymes for deconjugating and maturation. Specific SENPs have been observed to exhibit preferences for specific SUMO paralogs. The amino acids C-terminal are removed during the maturation process, which is required for conjugation. The amino acids C-terminal to the di-glycine motif control the substrate selectivity of SENPs processing [31]. SENP1 has been shown to have an involvement in both SUMO deconjugation and SUMO processing in biochemical experiments. According to the statistics, SUMO1 is processed rapidly, followed by SUMO2 and SUMO3. In contrast, SENP1 showed minimal selectivity with regard to the capacity to deconjugate the different SUMO paralogs from SUMOylated targets in vitro [283–285]. However, recent genetic evidence from mice suggests that SENP1 only plays a small role in SUMO2 and SUMO3 deconjugation in vivo but is required for the deSUMOylation of SUMO1-modified proteins [286]. SUMO deconjugation is catalysed by SENP2 more effectively than SUMO synthesis, according to biochemical experiments, and it is more productive than SUMO1 and SUMO3 [287–289]. The SENP3-SENP5 pair has a marked predilection for SUMO2 and SUMO3 processing and deconjugation, but no appreciable processing or deconjugation of SUMO1 [290, 291]. SENP6 and SENP7 were barely able to deconjugate SUMO from the substrate due to their limited action activity. However, notably, SENP6 and SENP7,

and the comparable Ulp2 from yeast, could disassociate SUMO molecules from substrates that were SUMO2/3 SUMOylated effectively.

3.2.3 The SUMOylation and deSUMOylation Process

SUMOylation is one type of protein multi-enzymatic Post-Translation Modifications (PTMs), and it is also a reversible and dynamic process. It is necessary for cellular processes, which is a significant regulator of protein function, activity, stability and intracellular localisation [211][216]. It has been reported that the cooperation between SUMOylation and deSUMOylation could regulate a varied spectrum of biological responses, such as transcription, cell division and signal transduction to carcinogenesis and viral replication. For many target proteins, i.e. mammalian cells under extreme environmental or metabolic stress conditions, the equilibrium between SUMOylation and deSUMOylation will be directly changed [28]; in addition, they also mentioned that this is a novel and emerging field of research to study the function of protein SUMOylation in health and diseases. For example, it has been shown that heat shock leads to changes in SUMO-2 conjugation and involves a wide range of cellular functions, including transcription, cell death and protein folding and degradation [36]. SUMOylation is associated with several diseases, including cancer, stroke and diabetes, as well as neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) [34][212]. Dramatic changes in global SUMO-2/3 conjugation in mammalian cells appear to have resulted from the changes in environmental temperature, metabolic stress, oxidative status, and oxygen availability [34][292]. Furthermore, SUMOylation of a specific proteome will be induced by specific stress, and these proteomes may be different among various organisms [214]. For example, in yeast, SUMOylation of DNA damage response proteins is primarily triggered by DNA damage, such as homologous recombination (HR) proteins [214]. By contrast, in human cells, the SUMO network is principally composed of chromatin modifiers, and transcription factors [5]. It should be noted that SUMOylation is a process that is fast reversible and may generate long-term effects [40].

The SUMOylation mechanism is shown in Figure 3.1. The SUMOylation pathway consists of three chemical reactions, which are associated with several specific enzymes (E1, E2, E3

and SENP). The SUMOylation status of a substrate protein is a dynamic balance between conjugation and deconjugation, and the pathway is relatively well characterised. Inactive precursor SUMO (pre-SUMO) is matured by SENPs to expose a di-glycine C-terminal motif and activated by an ATP-dependent E1 (activating) enzyme, formed by a heterodimer of SAE1 and SAE2. E1 passed the activated SUMO onto the only SUMO E2 (conjugating) enzyme, Ubc9, via a transesterification reaction, which catalyses SUMO conjugation, usually in conjugation with an E3 (ligating), which acts as an adaptor between Ubc9-SUMO and the substrate protein that mediates target selection and specificity. However, in some cases, SUMOylation can proceed without an E3 because Ubc9 binds directly to the consensus SUMOylation sequence to align the active site of Ubc9 directly next to the ε-amino group of the lysine residue to which the SUMO is to be conjugated.

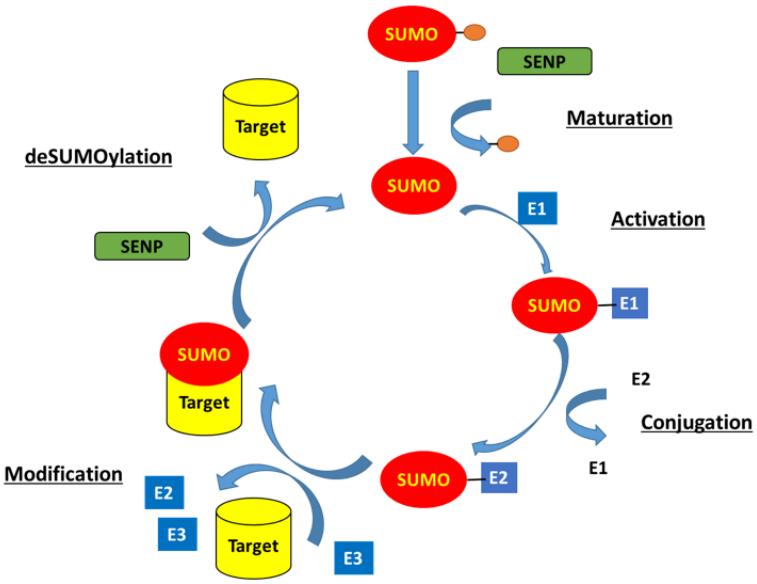

Figure 3.1. Biological mechanism of SUMOylation and deSUMOylation. Adapted from [243]

3.3 Role of SUMOylation in Cellular Response to Heat Shock

3.3.1 The Interaction Between SUMOylation and the Heat Shock Response

In recent years, SUMO has emerged as a critical player in proper cell homeostasis, owing to its ability to regulate transcription. In contrast, SUMO is critical for the response to cellular stress, and many physiological stressors result in increased in SUMO conjugate production. SUMO may bind to a wide range of proteins and control their fate, localisation and function [293]. Regulating transcription is a significant part of the SUMO stress response, and numerous pathways have been identified by which SUMO may regulate transcription. Despite the fact that several specific substrates become SUMOylated during the SUMO stress response, an emerging hypothesis is SUMO's alteration of the whole complexes, or pathways [214]. The main job of HSF1 is to activate genes that code for chaperones and other proteostasis factors, which are needed to deal with protein misfolding caused by high temperatures [294]. Because of the transcriptional nature of this response, there is a temporal lag between the quick start of proteotoxic stress and the availability of enough new chaperones to deal with the misfolded proteins [295].

The SUMO stress response causes enormous alterations in the proteome, implying that it impacts gene expression. Actively transcribed inducible genes contain SUMOylated proteins, such as heat shock genes [13][36][296]. On the one hand, heat shock causes SUMO2/3 to be modified, which upregulates genes involved in cell survival, growth, and death. SUMO2/3 modification, on the other hand, primarily suppresses transcription-related genes and decreases the total burden of protein quality monitoring systems when exposed to heat shock stress [9][296]. Therefore, SUMOylation has been hypothesized as a strategy for strictly controlling heat shock genes by limiting transcription hyperactivation [296].

Protein post-translational modifications are exceptionally well adapted to regulating cellular activities during stress reactions due to their dynamic character, which allows them to act transiently. The mysterious function of SUMO signalling in the HSR and a distinct group of HS-induced SUMO2/3 targets that the proteostasis network selectively regulates have been

discovered [297]. Given the rapid accumulation of SUMOylation after the commencement of HS and the widespread usage of SUMO as a linear fusion to recombinant proteins to improve solubility, it is reported that SUMOylation would be preventing aggregation in the early stages of HS [298, 299]. Protein stability is reduced by heat shock stress, affecting cellular structures and organs, including the nucleus [7]. Transiently produced heat shock proteins (HSPs), which operate as molecular chaperones for the native protein structure, and heat shock factors (HSFs), which modulate the heat-responsive genes transcription, are the most well-studied resistance proteins [300].

Furthermore, the phosphorylation and acetylation of HSF1 have been the subject of several researches. In response to HSR-induced stress, HSF1 is phosphorylated at several locations, which is assumed to be a sign of HSF1 activation [13][294][301]. Although SUMOylation has been extensively explored in the area of gene transcription regulation and HSR, the post-transcriptional modification of HSF1 with SUMO has received less attention [302–304].

When heat shock stress is applied, the SUMO stress response is triggered, resulting in the accelerated conjugation of SUMO2/3 to protein substrates [204][303][305]. The addition of SUMO to partially misfolded proteins is thought to be an early response to protein misfolding, preserving them by enhancing their solubility [297]. Heat shock sensitivity is consistently increased in cells lacking SUMO2/3 [36].

3.3.2 The Regulation of HSF Protein SUMO Modification

The transcription of heat shock genes is induced when cells are exposed to various environmental stimuli, such as heat shock. Heat shock transcription factor 1 (HSF1), which occurs in a non-DNA-binding form in the absence of stress, regulates the transcription of heat shock genes. HSF1 is converted to a trimeric DNA binding form by heat shock, which subsequently interacts with promoters of heat shock protein (HSP) genes to up-regulate transcription [306, 307]. It has been reported that heat shock stress causes HSF1 to become covalently modified by SUMO1 and that this modification stimulated HSF1's DNA binding ability [308].

HSF1 is a transcription factor that regulates the production of heat shock proteins in cells that have experienced heat shock stress. HSF1 gains DNA binding capacity and localises to

nuclear stress granules in response to stress, although the molecular mechanisms by which these processes are mediated remain unknown. The SUMO1 modifies HSF1 at lysine 298 in response to stress. Nevertheless, the biochemical mechanism behind the stress-induced SUMO1 modification of HSF1 protein remains unclear. HSF1 DNA binding complexes are supershifted by antibodies against SUMO1, and HSF1 is converted to the DNA binding form in a reconstituted SUMO1 reaction system. In nuclear stress cells, HSF1 colocalises with SUMO1, which is prohibited by a lysine 298 mutation. Stress-induced transcriptional activity of HSF1 is significantly reduced in vivo when lysine 298 is mutated [308].
HSF2, another HSF family member, has recently been discovered to be constitutively modified in vivo by SUMO1 [309]. As a result, this suggests that HSF1 is subjected to stress-regulated SUMO1 modification, which is responsible for the factor's conversion to the DNA binding form and/or localisation to nuclear bodies in response to stress. In contrast to HSF2, the HSF1 protein is not constitutively modified by SUMO1 and is only regulated when cells are subjected to stress conditions, according to the study. After stress treatment, HSF1 colocalises with SUMO1 in nuclear bodies, with kinetics that closely resemble those that of heat shock treatment and recovery. The location of stress-induced SUMO1 alteration in HSF1 has been identified as lysine 298. Stress-inducible HSF1 activation of HSP gene transcription is considerably reduced when this residue is mutated, suggesting that SUMO1 modification at this location is required for optimal stress-induced HSF1 activity. Moreover, it has implied that the DNA binding activity of HSF1 is regulated by SUMO1 modification through modulating its function [308].

3.4 Role of SUMOylation in Cellular Response to Hypoxia

3.4.1 The Interaction Between SUMOylation and the Hypoxia Response

Increasingly studies show that proteins must be covalently attached to SUMO for the hypoxia response and subsequent signalling cascade to function correctly. This might be due to alterations in the SUMO conjugation process causing modifications to specific proteins in the hypoxia signalling cascade or global SUMOylation levels in hypoxia cells [22][310]. Hypoxia is frequently seen at the tissue level as part of ischemia (blood flow

restriction), which is characterised by extreme hypoxia and nutrient depletion and can result in cell death in block regions. Ischemia has been shown to generate significant alterations in global SUMOylation and SUMO mechanism elements in animal hearts and brains. Following ischemia, SUMO conjugation is elevated globally as a stress-protective strategy, according to earlier studies [311, 312]. Characteristically, in the brains of rats with middle cerebral ischemia, a significant rise in SUMO was detected [313–315]. In comparison to wild-type mice, brain infarct sizes were reduced in a transgenic mouse model due to Ubc9 being overexpressed; consequently, a rise in SUMO1- and SUMO2/3-conjugated proteins as predicted [316].

Whereas modifications of the SUMOylation mechanism have been reported in brain tissue of ischemia animals, there are few findings on SUMOylation modification under hypoxia stress. Enhanced SUMO1 expression in colon cells cultivated during hypoxia, as well as in the heart and brain of mice given 10% oxygen, was the first evidence of increased global SUMO conjugation in response to hypoxia [38][317]. Based on the above, unique SUMOylation pathways are thought to define responses to hypoxia or ischemia irritant. Although the conjugation of SUMO2/3 is thought to be critical for cell survival under ischemia stress, regulation of SUMOylation is more insightful under hypoxia and could be utilised for physiological responses to oxygen deprivation a that the cellular system is well prepared for [42]. Emerging evidence indicated that SUMO could interact with and regulate a number of proteins involved in the hypoxia signalling pathway. Such modifications are required for their normal function. The influence of hypoxic process components by SUMOylation has been explored [39][161].

HIF transcription factors are the most critical participants in the hypoxia response pathway. Other post-translational modifications, including phosphorylation, ubiquitination and SUMOylation, govern the oxygen-sensitive HIF subunits besides hydroxylation. These alterations may have an effect on their protein stability as well as the transcriptional activity [310][318]. It has been mentioned that HIF1α can be SUMOylated at residues K391 and K477 [24], although the effects of these modifications are unknown. SUMOylation of HIF1α improves its stability, and transcriptional activity was increased after overexpression of SUMO1 in 293T cells [39]. In consensus, hypoxia stimulated the RWD-containing SUMOylation enhancer (RSUME) to improve the SUMOylation of HIF1α, which increased HIF1α stability and transcriptional activity [319].

3.4.2 The Role of SENP1 in the Stabilisation Activity of HIF1α in Hypoxia

It has been determined that SENP1 regulates the stability of HIF1α, which governs erythropoietin synthesis. Hypoxia causes SUMOylation of HIF1α, which leads to HIF1α destruction via a VHL and proteasome-dependent process. SENP1 deconjugates SUMOylated HIF1α, allowing it to survive hypoxia and avoid destruction. These findings show that SENP1 plays a crucial physiological function in the hypoxia response by regulating HIF1α stability and that SUMOylation may also cause a protein to be ubiquitinated and degraded [43]. Increasing research employing SUMOylation lacking HIF1α forms revealed that the absence of SUMO modification improved HIF1α transcriptional activity [24]. In agreement, Cheng *et al.* [43] proposed that SUMOylation of HIF1α elevated when SENP1 was removed, it degraded in a VHL-dependent way, whereas SENP1 expression was required for transcription activity of HIF1α.

SENP1 promoted the stability and transcriptional activity of HIF1α in hepatocellular carcinoma (HCC) and ovarian cancer cells by deSUMOylation in current findings. In kidney cancer cells, increased SENP1 expression was also linked to overexpression of glycolytic enzymes, which are recognised HIF1α targets. Furthermore, SENP1 is a significant target of HIF1α, implying the existence of a positive feedback cycle in which SENP1 expression increases HIF1α activity has been shown [320, 321]. The seeming dispute over HIF1α SUMOylation may be observed that while the stability in the SUMO mechanism factors expression such as SENP or SUMO is disrupted, it affects not only HIF1α but also several other elements of the hypoxia pathway at the same time. Depending on the cellular environment, this can lead to a wide range of consequences (in connection with HIF1α activity). SUMOylation has been shown to affect the activity of several different proteins that are not directly involved in the hypoxia signalling pathway, in addition to the primary regulators of the hypoxia process.

In a word, SUMOylation of protein is involved in practically every aspect of cell life. More critically, all evidence suggests that SUMOylation and deSUMOylation are a dynamic machinery in the cell response to hypoxia condition. While much progress has been achieved in understanding the significance of SUMOylation in hypoxia circumstance, there is still enormous debate and many open problems. Given that hypoxia is a core of severe

pathological conditions, a critical future goal will be to understand better the interactions among SUMOylation and the necessary pathways of the hypoxia response process so that they can be controlled, and novel molecular interventions can be developed. Considering novel developing compounds that target SUMOylation have shown potential in treating human cancers [322]; they are also regarded as ideal for modifying the SUMO mechanism and showing therapeutic value in hypoxic tumours.

3.5 SUMOylation and Human Diseases

3.5.1 SUMOylation and Neurodegenerative Disease

All signalling pathways and regulatory systems that allow cells to adapt to changing environmental circumstances rely on post-translational changes. For sustaining neuronal cell survival, function, and connection, they must be tightly controlled in the central nervous system. While other post-translational modifications, particularly phosphorylation and ubiquitination, have long been studied in relation to neuronal function and dysfunction, SUMOylation of neuron-specific targets has been unknown until recently, as most SUMO conjugated protein assays have been performed in non-neuronal cell lines. Moreover, due to this protein modification's unstable and difficult-to-detect nature, covalent SUMO modification did not become known until 1996. Nevertheless, the growing evidence of the significance of SUMOylation in diverse cellular processes has promoted plenty of research looking at elucidating SUMO's role in the nervous system [46].

Neurodegenerative diseases are defined by gradual and broad neuronal loss in particular groups of neurons and brain regions, resulting in the clinical symptoms seen. The accumulation and aggregation of misfolded proteins, considered a critical step in the disease pathogenesis, is one of the most prevalent features of these disorders. The component proteins of the inclusions and cellular position the allocation of the deposits dictate the histopathological characteristics of each disease. The role of these aggregated proteins in neuronal death has been contested, with some suggesting that huntington nuclear deposition might be neuroprotective [323, 324]. Nevertheless, in Alzheimer's disease, the resolvable oligomeric types of the amyloid-β (Aβ) peptide are likely to constitute the principal cytotoxic species [325, 326].

Alzheimer's disease (AD) is a chronic neurodegenerative disease among the elderly that usually starts slowly and gradually worsens over time, and about 35.6 million people were diagnosed with this disease around the world until 2010 [327]. More importantly, it is predicted that this number will almost double every 20 years, reaching 65.7 million in 2030 and 115.4 million in 2050. [328]. AD is a cause of 60-70% of cases of dementia and is considered to be multifactorial neuropathology [329]. AD is a disease caused by protein misfolding and through abnormal accumulations of the microtubule-associated protein tau and amyloid-β (Aβ) in certain brain areas [46][330].

Alzheimer's disease is now regarded as a multifaceted neurological disorder whose complexity is the reason for our slow progress in understanding the underlying processes and finding a possible definitive solution. Doctors are cognizant of the limited medical options for curing patients with AD, restoring their memory, and returning them to everyday life. However, the treatment technologies today are unable to differentiate between pathologies [329]. Today, only symptomatic treatments are available for AD, and consequently, uncovering novel molecular mechanisms underlying AD would be crucial for the development of treatment for AD. Due to the limited therapy methods, in recent years, research has tended to establish new theories that might aid in the development of future beneficial treatments. It has been suggested that SUMOylation is implicated in the molecular processes of numerous neurodegenerative diseases, including AD. Surprisingly, SUMOylation is a pathological symbol in AD and other neurodegenerative diseases [331]. Indeed, the imbalance between SUMOylation and deSUMOylation seems to be associated with the transition of numerous proteins implicated in AD pathogenesis from regular to pathogenic behaviour [329]. Although it is yet unknown how SUMOylation is correlated to AD, it has recently been shown that various proteins essential in the disease, such as Aβ and tau, are SUMOylated [332].

The impact of SUMO on particular AD proteins is discussed. Amyloid precursor protein (APP) and protein tau, which are associated with AD, have been identified as targets for SUMOylation. SUMO1 has been shown to modify tau on lysine K340, a location of tau-microtubule interaction. It is debatable if SUMO has a role in tau aggregation [333]. APP can be SUMOylated in vitro by SUMO1 and SUMO2/3, and SUMOylation seems to decrease Aβ aggregates. Moreover, it has also been suggested that SUMOylation could regulate APP, tau, and other proteins related to AD [329]. It has been concluded that

several proteins fundamentally crucial for the development of AD are SUMO targets, and compared with age-matched control individuals, the level of SUMO1-modified proteins in the cortex of AD proteins is increased [334].

Parkinson's disease (PD) is among the most typical degenerative central nervous system diseases that cause the motor and non-motor symptoms. In instances with PD, immunoreactivity to the SUMO was discovered, among others. SUMO-modified proteins are found in many disease-related proteins, and this post-translational modification has been related to neurodegeneration. In recent years, SUMOylation and SUMO-mediated mechanisms have been extensively explored, revealing nuclear and extranuclear activities for SUMO in some cellular processes, including transcriptional activity control, signal transduction pathway modification, and cellular stress response. This suggests that SUMO serves a purpose other than ubiquitin and proteasomal degradation. The discovery of risk and age-at-onset gene loci was a milestone in PD research, advancing our understanding of the disease's molecular processes. Mitochondrial dysfunction and poor mitochondrial quality control have become increasingly connected to PD. SUMO is engaged in several of these activities and is regulated in cellular stress response, underlining the worth of SUMOylation in physiology and pathology [335].

PD is the second most prevalent neurodegenerative condition, defined by cardinal motor symptoms such as stiffness, bradykinesia, or rest tremor, which result from dopaminergic neuron loss. Non-dopaminergic degeneration also occurs, and it appears to be the cause of the olfactory, emotional, and memory abnormalities that emerge before the characteristic motor signs of PD. Even though most PD cases are sporadic, multiple genes have previously been linked to the disease's hereditary manifestations. SUMO, a post-translational modification that affects a range of cellular processes, modifies the proteins produced by several genes, including α-synuclein, DJ-1, and parkin. Mitochondrial dysfunction is one of the pathogenic pathways causing PD. Recent research suggests that SUMOylation might be involved in mitochondrial dynamics, which are critical for brain function, and may play a vital role in the aetiology of PD [210][335].

In addition, Huntington's disease and other polyglutamine diseases have all been found to have SUMO proteins within inclusions. SUMO targets have also been found in a number of proteins implicated in various neurological disorders. Considering that the cellular processes are regulated by SUMO modification, there is a range of potential pathways

whereby SUMOylation might participate in the fundamental neurodegenerative processes [336].

3.5.2 SUMOylation and Cancer

Considering SUMOylation is required by almost cells, mutations of function loss in SUMO pathway elements cannot be a cause of cancer. Therefore, the fundamental SUMO modification process for genetic integrity is not impaired in cancer [337]. Many of the malignancies studied appear to have increased production of SUMO-activating enzyme E1, SUMO-conjugating enzyme E2, or SUMO-ligating enzyme E3. Although this may not always be proven in this research, that is logical to suppose that higher levels of SUMO, E1 or E2 enzyme correspond to higher SUMOylated protein levels. Furthermore, higher SUMO or SUMOylation enzyme levels when these cells are stressed allow them to increase SUMO conjugation levels. Admittedly, in many situations, increased SUMOylation capacity is associated with a worse prognosis [338]. The discovery that was temporarily inhibiting SUMO2 improves the efficacy of reprogramming to induced pluripotency acquisition, which may be especially relevant in cancer. Therefore, enhanced SUMOylation should be considered as an 'enabler' or 'protector' of cancer rather than a 'driver' or 'passenger'. Cancer cells may potentially expose particular weaknesses not shared by healthy cells if SUMOylation is absent or inadequate [47].

Evidence suggests that a significant function of SUMOylation in cancer is increasing, and it is associated with the essential role of SUMOylation in preserving chromosomal integrity and controlling cell proliferation. Many SUMO machinery elements are significantly expressed in cancer tissues, implying that active SUMOylation is associated with tumour formation. Many kinds of cancer, including ovarian [339], colon, and prostate cancer [215], have overexpression of the SUMO conjugating enzyme Ubc9, which promotes cell invasion and metastasis [340]. Surprisingly, malignant cancers have a high level of numerous SUMO proteases, including SENP1 and SENP5, suggesting that SUMOylation must be strictly controlled to avoid malignant development and cell proliferation [341]. Notably, knocking out the SUMO E1 subunit SAE2 in mice significantly reduced colon tumour development, demonstrating the significance of SUMOylation tumour formation [342].

Although cancer could be triggered by inheritance and mutation of tumour suppressors and unnatural enhancement in oncogene at the genetic level, emerging evidence shows that cancer progression is mainly regulated by PTMs such as SUMOylation. A significant contribution is believed to be made by SUMOylation through regulating critical elements and signalling processes. SUMOylation mechanisms can be dynamically regulated under diverse stresses to produce persistent potentials [343]. Several lines of evidence suggest that SUMOylation-mediated pathways could act as potential therapeutic targets in cancer [344]. Currently, some studies have indicated that expression of SUMO E1 activating enzyme (SAE1, SAE2), E2 conjugating enzyme (Ubc9) and E3 ligase (PIASy) increased in occurrences of many cancer types. For instance, the increased levels of E1 enzyme are associated with the hepatocellular carcinoma (HCC). In addition, the upregulated Ubc9 levels are found in a few types of human cancers, including adenocarcinoma and ovarian cancer. Cancer cell growth can be enhanced by overexpression of Ubc9 [339][345], and PIASy is improved in various cancer types, such as lung cancer, breast cancer, prostate cancer, and colorectal cancer [47]. Furthermore, the expression levels of SENP are increased in prostate, and thyroid cancer [346, 347]. The relationship between SUMOylation or deSUMOylation and cancer is complicated; therefore, there is still a long way to go in exploring potential correlations. Moreover, while the SUMOylation-deSUMOylation equilibrium is maintained and regulated to a certain extent, how disease conditions lead to imbalance in SUMOylation and deSUMOylation remains unknown.

3.5.3 SUMOylation and Transient Cerebral Ischaemia

It is well known that the activation of SUMOylation of target proteins depends on various stresses, including heat shock stress, hypoxia stress, oxidative stress, and metabolic stress [38][151]. It has been suggested that the numerous processes of cells are affected by ischaemia, which is an extreme metabolic stress [28]. It has been discovered that ischaemic stress was shown to increase the levels of SUMO conjugation, especially SUMO-2/3, generally, during reperfusion in animal models and the restoration of oxygen and glucose in mammalian cell culture systems [348]. Except for the shift in SUMO-2/3, the rapid decrease in SENP3 levels during ODG (oxygen/glucose deprivation) would promote cell survival [349]. Similarly, it is suggested that transient brain ischaemia will be involved

in a sharp increase in the level of SUMO-conjugated proteins in hibernation. With the developed detection of novel brain injury mechanisms and the continued failure of clinical trials of cell-based therapies for a single mechanism, ischaemic brain injury is increasingly seen as a highly complex multifactorial process involving the interaction of many non-dominant effectors [312][348]. It is reported that transient cerebral ischaemia includes an extensive increase in protein SUMO-2/3-ylation in both the hippocampus and cerebral cortex [311]. The enhancement of SUMO-2/3 conjugation seemed to be correlated to a reduction in levels of free SUMO-2/3. There are different changes in protein levels of the SUMO-conjugating enzyme Ubc9 in the cortex and hippocampus after ischaemia: the levels decreased in the cortex but did not change in the hippocampus. However, the reason for the different changes in levels between Ubc9 and SUMO-2/3 in the cerebral cortex after ischaemia is unclear. Hence, a question is posed whether or not changes in SUMO-2/3 conjugation and SUMO-conjugating enzyme (Ubc9) are consistent under ischaemia conditions. Ischaemia included changes in protein modification by SUMO-1 that seem to differ from that by SUMO-2/3. There is no significant change in the SUMO-1 conjugation, while SUMO-2/3 modification is massively increased after ischaemia [315]. This implies that the cellular response to metabolic stress (in both the cortex and hippocampus) is dependent on SUMO-2/3 conjugation.

3.5.4 SUMOylation and Oxidative Stress

SUMOylation is regarded as an “oxidative stress sensor” as global SUMOylation increases while the homeostasis of redox status rises in the cells and reactive oxygen species (ROS) production is promoted [206]. Some research looked at how oxidative stress affects both SUMO machinery and SUMOylation activity, result in conflicting findings. Indeed, variable SUMOylation modulation corresponds to distinct levels of cellular ROS. High concentrations of ROS (100mM H_2O_2) cause SUMO conjugation to rise, whereas physiological levels (1mM H_2O_2) lead SUMO deconjugation to occur in a time-dependent manner [249]. Other investigations have shown that oxidative stress can reduce SUMOylation, although low amounts of ROS (0.5mM H_2O_2) increased in a different cellular model [350].

Oxidative stress is a series of adaptive reactions caused by the imbalance between the

active oxygen component and the antioxidant system [332][351]. Through interfering with the normal redox state of cells, it produces peroxides and free radicals that cause toxic effects, thus damaging the proteins, lipids and DNA of the cells. Under conditions in which mitochondrial metabolism produces energy, cells produce oxidizing substances to maintain normal processes. Cells require a well-balanced biological process to maintain the physiological homeostasis of normal tissue [332]. For humans, oxidative stress is implicated in many human diseases, which is why it is so ominously important. For example, oxidative stress is generally associated with PD [352], AD [353], cancer [354], diabetes [239] and heart failure [351].

AD is thought to be triggered by oxidative damage, which is one of the reasons. It is important to note that the N-terminal metal-binding domains of both APP and Aβ have binding sites for copper and zinc and that oxidative stress causes Aβ at least in part [355]. Iron, copper, and zinc, which are often heavily concentrated inside the core and periphery of Aβ deposits, have been demonstrated to create hydrogen peroxide by metal ion reduction and can boost the production of free radicals by Aβ [356]. Considering SUMOylation plays a crucial role in oxidative stress response and AD progression, several SUMO targets implicated in oxidative stress might be explored as novel therapeutic targets in Alzheimer's disease.

The balance of the cellular oxidation system is regulated by the dynamic process between the formation and removal of oxidative free radicals. The cellular oxidation system can be divided into two broad categories: reactive oxides species (ROS) and reactive nitrogen species (RNS) [332]. In organisms suffering from oxidative stress, many different changes in biological processes occur. For instance, protein SUMOylation is a typical example. Induction of oxidative stress can enhance the global protein SUMO conjugation [206][332]. It is claimed that following oxidative stress and alcohol treatment, there is a rapid change of most SUMO-2/3 from the unconjugated form to the conjugated form. The protein of SUMOylation in cells appears to be increased by an extreme oxidative stress condition (H_2O_2 at 100mM). In contrast, treating cells with a lower concentration (1mM) of H_2O_2, decreases the protein of SUMOylation dramatically. Therefore, the changes of SUMO conjugation seem to depend on the concentration of H_2O_2, i.e., a reactive oxygen species to induce oxidative stress directly. When the concentration of H_2O_2 is high, the SUMO conjugation increases and vice versa [249]. These changes in SUMO conjugation

and deconjugation indicate that the conjugation of SUMO is affected by the H_2O_2 dose. Under a low concentration of H_2O_2, only the SUMO conjugation is affected, resulting in accumulation and free SUMO. While at higher concentrations, both SUMO conjugation and deconjugation are severely impaired. Due to the process of SUMO, conjugation and deconjugation is a reversible cycle. Hence, the reduction of the SUMO conjugation can be caused by a fast reversible deconjugation, and most proteins are unbound in a short time. This result also demonstrates that low concentrations of H_2O_2 can cause deSUMOylation of most substrates. In addition, oxidative stress may cause a thioester bond formation between Uba2 and Ubc9, which is responsible for the inactivation of SUMO conjugation [249].

In fact, SUMOylation and deSUMOylation are essential factors for the cellular response to stresses, such as heat shock, hypoxia, ischaemia and oxidative stress. Obviously, there are various enzymes involved in these processes. However, it is still uncertain whether changes in SUMO-2/3 conjugation and SUMO conjugating enzyme (Ubc9) are consistent or not under all conditions, for instance, in transient cerebral ischaemia stress. Moreover, these findings have raised the question of how the stress signal is transduced to lead to alterations in SUMOylation. Furthermore, despite the fact that the equilibrium between SUMOylation and deSUMOylation is maintained and regulated to a certain degree, how disease conditions lead to an imbalance between SUMOylation and deSUMOylation is largely unclear.

3.5.5 Summary

SUMOylation is a crucial step in the production of proteins that are connected to a variety of biological functions. It is well accepted that cellular stress results in worldwide increases in protein SUMOylation. It has recently become evident that exposure to environmental or metabolic stress directly modifies the balance between SUMOylation and deSUMOylation for many substrate proteins. Due to its ability to control transcription, SUMO has recently been recognised as a crucial component of healthy cell homeostasis. However, SUMO is essential for the cellular stress response, and most physiological stresses increase SUMO conjugate formation. In addition, many different proteins may bind to SUMO, which can then influence those proteins' function, localisation, and fate [293]. Studies increasingly

demonstrate that the proper operation of the heat shock or hypoxia response and subsequent signalling cascade depends on proteins covalently linked to SUMO. Moreover, much research to clarify the function of SUMO in the nervous system has been made possible by mounting evidence demonstrating the significance of SUMO acylation in many cellular processes [47]. The significant function of SUMOylation in human diseases is growing, and it is linked to cancer, transient cerebral ischaemia and neurodegenerative diseases [47][315][337].

Although the available information for many individual proteins is imprecise and frequently contradictory, global SUMOylation is a vital cell destiny predictor. Consequently, despite the considerable advancements, much more research is required to fully comprehend the intricate control, target, and consequences of the deSUMOylation enzyme. In addition, considering the critical role of mathematical models in many scientific and engineering disciplines and considering the importance of a better understanding of the role of SUMOs and SUMOylation in the sensing, responding, and defending mechanisms of human cells to the extreme environment and pathological conditions, it is highly necessity of having a comprehensive and accurate mathematical model to describe such a dynamics process, which described in Chapter 4 and Chapter 5.

Chapter 4

Dynamic Modelling of SUMOylation in Cellular Response to Heat Shock

4.1 Introduction

Despite the roles of the processes of HSR being well documented, the intricacies of the HSF1 activation attenuation cycle remain elusive at a molecular level. One of the key mechanisms proposed to adapt the HSF1 activity to the needs of individuals cells is post-translational modifications (PTMs) [28][294]. While phosphorylation of HSF1 at multiple sites has been studied intensively [13][301][357], only recently experimental evidence has suggested that SUMOylation as one PTM type is a key determinant of cell fate in response to these extreme stresses, including HS stress [9][28, 29]. As mentioned above, SUMOylation is a dynamic PTM process found in all eukaryotes where small ubiquitin-like modifier (SUMO) proteins, are covalently attached to target proteins in cells to modify their functions. However, how SUMOylation affects HSF1 activity at a molecular level is still unclear. It has been implicated in the regulation of a host of cellular processes, and is essential for health, and even the survival, of most organisms [30]. In other words, SUMOylation triggered by cellular stresses such as HS stress could be a survival mechanism for cells in response to various stimuli to survive. Current evidence shows that increased SUMO-2/3 conjugation following HS is essential for cell survival [35, 36]. In addition, experimental studies conducted in mice have revealed that the accumulation of misfolded proteins, most likely following dysfunctional cell stress responses, is associated with age-related diseases

including Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) as well as cancers [46, 47][358–360]. Therefore, a better understanding of the HSR pathway and SUMOylation regulatory machinery is imperative, and it would lead to the development of new therapeutic strategies for preventing and/or treating those diseases associated with cellular stress. In this chapter, a combined mathematical modelling and experimental approach to investigating the role of HSF1 SUMOylation in response to HS will be described.

Compared with experimental studies of HSR, there has been some progress [4][16–18] in its mathematical modelling since seminal work has been published in [15]. The computational results of the models [4] showed that, for the administration of the HS, a significant rise in HSP synthesis was initially observed and followed by a slow decrease back to its original steady-state value. In the case of slow-heating, only a small rise in the synthesis of HSF was observed, i.e., almost no changes in the original level. In case of a short HS the same significant rise in HSP synthesis was initially observed as in the case of a long HS, but the following decrease in HSP synthesis was faster. In [16], the authors proposed a data-driven dynamical model of the heat shock response in *Chlamydomonas reinhardtii*, where a Monte Carlo (MC) scan of the parameter space was performed to gain insight into its structure and then a gradient search was used to find a set of parameters which locally optimizes the difference between the model simulations and experimental data. The final model was validated with independent experimental results extracted from the literature for certain behaviours. All these HSR models provide some degrees of insights into the mechanism of the HSR.

As the new experimental evidence emerged recently, it is important to update these existing models by augmenting them with PTM processes, in particular, the SUMOylation of HSF1. To this end, on the one hand, a dynamical model is presented to simulate the role of HSF1 SUMOylation in response to HS. On the other hand, an experiment is conducted for the purpose of validation of the proposed model in terms of the SUMOylation levels under the HS. Key features of this model are inclusion of HSR, SUMOylation of HSF1, and heat shock protein (HSP) synthesis at a molecular level, describing the dynamical evolution of the key variables involved in the regulation of HSPs. To the best of our knowledge, this is the first attempt to investigate the HSR associated with SUMOylation of HSF1 by combining experiment and mathematical modelling. The biological process

of phosphorylation and SUMOylation of HSF1 in response to heat shock stress (block 4 and 5 in Figure 4.1) is proposed by the author, which is the innovation of the model. Subsequently, differential evolution (DE) and global sensitivity analysis are applied to the model for parameter optimisation and estimation.

The chapter is organised as follows. In Section 4.2, the new mathematical model is provided of the HSR of the cells considering SUMOylation of HSF1 based on the underlying signalling network inferred from experimental findings. In Section 4.3, a differential evolution (DE) algorithm is used to optimise the model parameters, and global sensitivity analysis is applied to display the influence of input factors and output variables. In Section 4.4, the experiments to validate the model are also presented and employ the model to stimulate interesting scenarios that have not yet been tested experimentally. Finally conclusions and future work are demonstrated in Section 4.5.

4.2 Model Description

Since SUMOylation influences the response of the cells to heat shock as discussed in the previous section, mathematical models of heat shock response should consider it explicitly, in this way providing additional opportunities to deepen the scientific understanding of the biological mechanisms at hand. In what follows, the detailed mechanisms and corresponding mathematical models will be discussed.

4.2.1 Model Overview—Biological Mechanism of Heat Shock

The new heat shock model is based on the underlying SUMOylation signalling network inferred from experimental findings, e.g. [28, 29] and previously published models of heat shock response *e.g.* [4][361], and consists of (i) at molecular level, the aggregation of the denatured proteins (DPs) upon HS and the chaperone activity of HSP (Block 1 in Figure 4.1), (ii) the activation of HSF1 and its trimerization (Block 2 and Block 3 in Figure 4.1), (iii) the phosphorylation and translocation of HSF1 trimers into nucleus and the subsequent SUMOylation (Block 4 in Figure 4.1), and (iv) the synthesis of HSPs regulated by HSF1 binding to HSE in the promoter of heat shock genes and stimulating their transcription (Block 5 and Block 6 in Figure 4.1). The resulting framework, which consists of ordinary

differential equations (ODEs) at the molecular level, is built and suitable to answer the research question at hand, i.e. the investigation of the role of SUMOylation in the cell HSR. A schematic overview of the model is given in Figure 4.1.

In response to HS, HSPs, as molecular chaperones are directed towards the capture of folding intermediates to prevent misfolding and aggregation of proteins, and to facilitate refolding or degradation in Block 1. Block 2 and Block 3 show the processes of HSF1 are activated, trimerized and phosphorylated. HSF1 trimers are translocated into the nucleus and subsequently SUMOylated in Block 4. Block 5 and 6 describe the HSPs synthesis is up-regulated by HSF1 binding to HSE in the promoter of HS genes and stimulating their transcription.

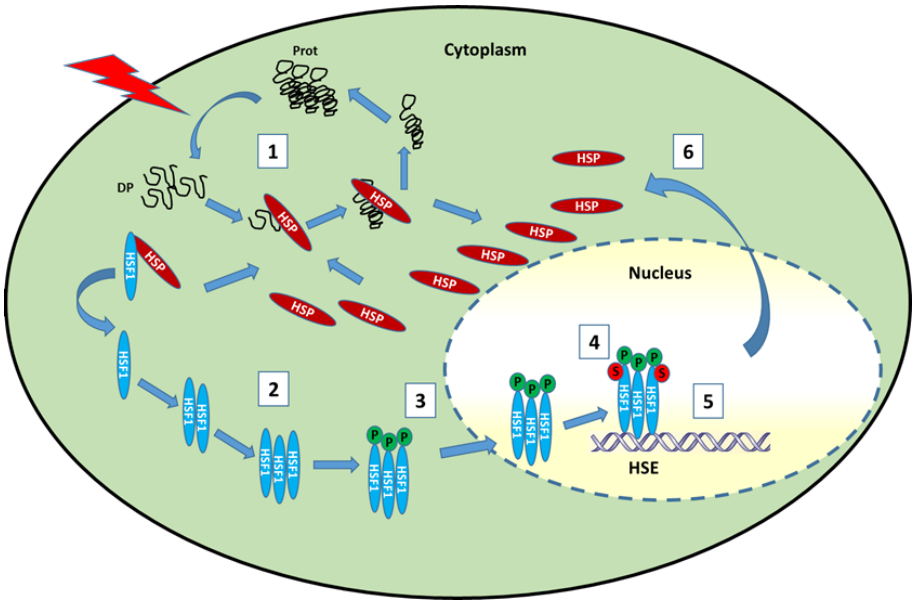

Figure 4.1. Biological mechanism of the cell response to heat shock stress – p: phosphorylation and s: SUMOylation.

One of the most well-known models of enzyme kinetics in biology is the Michaelis-Menten kinetics. A mathematical model of the reaction is proposed by [362] in 1913. It includes an enzyme E, forming a complex ES, with a substrate S and releasing a product P, which regenerates the original enzyme. This can be diagrammatically expressed as

$$[\mathrm{E}] + [\mathrm{S}] \underset{\mathrm{k}_r}{\overset{\mathrm{k}_f}{\rightleftharpoons}} [\mathrm{E}\cdot\mathrm{S}] \xrightarrow{\mathrm{k}_{\mathrm{cat}}} [\mathrm{E}] + [\mathrm{P}] \tag{4.1}$$

where k_f denotes forward rate constant, k_r means reverse rate constant and k_{cat} shows the catalytic rate constant.

A set of four nonlinear ordinary differential equations that specify the rate of change of reactants with time t are produced by using the law of mass action, which provides that the rate of a reaction is proportional to the product of the concentrations of the reactants (i.e. [E][S]) [363].

The reaction can be described with four differential equations

$$\frac{\mathrm{d}[E]}{\mathrm{d}t} = -k_f[E][S] + k_r[E \cdot S] + k_{cat}[E \cdot S] \tag{4.2}$$

$$\frac{\mathrm{d}[S]}{\mathrm{d}t} = -k_f[E][S] + k_r[E \cdot S] \tag{4.3}$$

$$\frac{\mathrm{d}[E \cdot S]}{\mathrm{d}t} = -k_f[E][S] - k_r[E \cdot S] - k_{cat}[E \cdot S] \tag{4.4}$$

$$\frac{\mathrm{d}[P]}{\mathrm{d}t} = k_{cat}[E \cdot S] \tag{4.5}$$

Block 1 shows the healthy protein *Prot* is converted into denatured protein *DP* when the temperature increased, and then the heat shock protein *HSP* as chaperones binds to the denatured protein *DP* to form *HSP·DP* complexes for the purpose of protecting and repairing. The reactions are described as follows

$$[\mathrm{HSP}] + [\mathrm{DP}] \underset{\mathrm{k}_{1r}}{\overset{\mathrm{k}_{1f}}{\rightleftharpoons}} [\mathrm{HSP} \cdot \mathrm{DP}] \tag{4.6}$$

Under physiological conditions, most of the *HSF1* exist in cells in an inactive form and bind to *HSP* to form an *HSP·HSF1* complex. However, under stress conditions, the monomer and dimer of *HSF1* are converted into active form $HSF1_3$. The block shows the binding of *HSP* to monomer form (*HSF1*)

$$[\mathrm{HSP}] + [\mathrm{HSF1}] \underset{\mathrm{k}_{2r}}{\overset{\mathrm{k}_{2f}}{\rightleftharpoons}} [\mathrm{HSP} \cdot HSF1] \tag{4.7}$$

and the interaction between monomer (*HSF1*), dimer ($HSF1_2$) and trimer form ($HSF1_3$) is described in Reaction (4.8)

$$3 \cdot [\mathrm{HSF1}] \underset{\mathrm{k}_{3r}}{\overset{\mathrm{k}_{3f}}{\rightleftharpoons}} [\mathrm{HSF1}_3] \tag{4.8}$$

Reaction (4.9) describes the translation of HSP

$$[\text{mRNA}] \xrightarrow{k_{mh}} [\text{mRNA}] + [\text{HSP}] \tag{4.9}$$

Degradation of complex HSP· mRNA is indicated in Reaction (4.10)

$$[\text{HSP} \cdot \text{mRNA}] \xrightarrow{k_m} [\text{mRNA}] + [\text{HSP}] \tag{4.10}$$

Reaction (4.11) demonstrates that trimers of HSF1 ($HSF1_3$) bind to HSE, which is a promoter region for genes transcription

$$[\text{HSF1}_3] + [\text{HSE}] \underset{k_{5r}}{\overset{k_{5f}}{\rightleftharpoons}} [\text{HSF1}_{3.s3}] \cdot [\text{HSE}] \tag{4.11}$$

The complex HSP·HSF1 as chaperones binds to the denatured protein DP to form complex HSP·HSF1·DP complexes for the purpose of protecting and repairing is described in Reaction (4.12)

$$[\text{HSP} \cdot \text{HSF1}] + [\text{DP}] \underset{k_{6r}}{\overset{k_{6f}}{\rightleftharpoons}} [\text{HSP} \cdot \text{DP}] + [\text{HSF1}] \tag{4.12}$$

Reaction (4.13) shows the synthesis of HSP·DP refolding process to release healthy protein

$$[\text{HSP} \cdot \text{DP}] \xrightarrow{k_{ref}} [\text{HSP}] \tag{4.13}$$

The degradation of HSP is displayed in Reaction (4.14)

$$[\text{HSP}] \xrightarrow{d_2} [\phi] \tag{4.14}$$

Reaction (4.15) demonstrated that mRNA transcription of $HSF1_{3.s3}$·HSE

$$[\text{HSF1}_{3.s3} \cdot \text{HSE}] \xrightarrow{k_t} [\text{HSF1}_{3.s3} \cdot \text{HSE}] + [\text{mRNA}] \tag{4.15}$$

In this process, the trimer form ($HSF1_3$) is as target protein and modified by the SUMO protein. The SUMOylation pathway consists of three specific chemical reactions, which is associated with several specific enzymes (*e.g. E1, E2* and *SENP*), and will be discussed in some detail below. In the model, the effect of enzyme *E3* is not considered. There is

no protein synthesis or degradation in some intermediate products (*e.g. presumo·SENP, sumo·E1, sumo·E2, SENP·HSF1*$_{3.s3}$).

Reaction (4.16) shows that the inactive sumo precursor (presumo) is cleaved by specific enzyme SENP, and converts the presumo into a mature form, the presumo·SENP is protein synthesis in the pathway

$$[\text{presumo}] + [\text{SENP}] \underset{\text{k}_{7r}}{\overset{\text{k}_{7f}}{\rightleftharpoons}} [\text{presumo} \cdot \text{SENP}] \xrightarrow{\text{k}_{7cat}} [\text{sumo}] + [\text{SENP}] \tag{4.16}$$

$$[\text{HSF1}_{3.s3}] + [\text{SENP}] \underset{\text{k}_{11r}}{\overset{\text{k}_{11f}}{\rightleftharpoons}} [\text{SENP} \cdot HSF1_{3.s3}] \xrightarrow{\text{k}_{11cat}} [\text{sumo}] + [\text{SENP}] + [\text{HSF1}_3] \tag{4.17}$$

Reaction (4.18) shows the activation process, sumo is activated by enzyme E1 and forms the complex of sumo·E1. It is an irreversible reaction, and k_8 means the rate constant

$$[\text{sumo}] + [\text{E1}] \xrightarrow{\text{k}_8} [\text{sumo} \cdot \text{E1}] \tag{4.18}$$

The reaction (4.19) reveals the conjugation step of SUMOylation process, the complex of *sumo·E1* and enzyme E2 binding together and forms synthesis sumo·E2 and enzyme E1 released finally

$$[\text{sumo} \cdot \text{E1}] + [\text{E2}] \xrightarrow{\text{k}_9} [\text{sumo} \cdot \text{E2}] + [\text{E1}] \tag{4.19}$$

The reaction (4.20) indicates the ligation step in SUMOylation, sumo·E2 binds to unmodified target protein HSF1_3, and forms SUMOylated target protein $\text{HSF1}_{3.s3}$, E2 is released at the same time

$$[\text{sumo} \cdot \text{E2}] + [\text{HSF1}_3] \xrightarrow{\text{k}_{10}} [\text{HSF1}_{3.s3}] + [\text{E2}] \tag{4.20}$$

4.2.2 The Mathematical Model of Heat Shock

The increase in the concentration of denatured proteins DP due to the rise of temperature *F(T)* used in this study is considered proportional to the protein denaturation rate φ_T, where T is the temperature and a function of time (t). A few proteins known as heat-shock proteins are synthesised selectively and quickly when cells are exposed to high temperatures. The response of a cell to a rise in temperature is closely correlated with fundamental genetic

networks, including cell cycle and metabolism. The protein denaturation rate φ_T depends exponentially on temperature and can be calculated by applying differential scanning calorimetry with the temperature rising steadily, in the range of $37\,^{\circ}\mathrm{C} - 45\,^{\circ}\mathrm{C}$ based on [3] and [15]. Where $[*]$ denotes the concentration of $*$, as a function of time.

$$\varphi_T = (1 - \frac{0.4}{e^{(T-37)}}) \times 1.4^{(T-37)} \times 0.3 \tag{4.21}$$

and

$$F(T) = 10 \times \varphi_T \tag{4.22}$$

The law of mass action to the previously described biochemical reactions is applied to obtain the system of ordinary differential equations (ODEs). The increased temperature will cause a change in the confirmation of cellular proteins. The protein is converted into denatured protein DP when the temperature is increased, and then the heat shock protein DP when the temperature is increased, and then the heat shock protein (HSP) as chaperones bind to the denatured protein DP to form HSP·DP complexes for the purpose of protecting and repairing. As indicated in Block 1 in Figure 1, this process involves two ways where one is the binding of HSP to DP and the other is the release of the HSF1 monomer. The dynamics of the denatured protein DP is thus as follows

$$\begin{aligned} \frac{\mathrm{d}[DP]}{\mathrm{d}t} =& F(T) - k_{1f}[HSP][DP] + k_{1r}[HSP \cdot DP] \\ & - k_{6f}[HSP \cdot HSF1][DP] + k_{6r}[HSP \cdot DP][HSF1] \end{aligned} \tag{4.23}$$

The increase in the concentration of denatured proteins DP due to the rise of temperature is denoted by *F(T)*, where T is the temperature. The term $[HSP][DP]$ represents the binding of HSP to DP while $[HSP{\cdot}DP]$ illustrates the dissociation of the HSP·DP complexes to free DP. The term $[HSP{\cdot}HSF1][DP]$ denotes the binding of HSP to DP and the release of the HSF1 monomer, and $[HSP{\cdot}DP][HSF1]$ expresses the reverse process. The dynamics of the formation of the HSP·DP complexes are described as (Block 1 in Figure 4.1)

$$\begin{aligned} \frac{\mathrm{d}[HSP \cdot DP]}{\mathrm{d}t} =& k_{1f}[HSP][DP] - k_{1r}[HSP \cdot DP] - k_{ref}[HSP \cdot DP] \\ & + k_{6f}[HSP \cdot HSF1][DP] - k_{6r}[HSP \cdot DP][HSF1] \end{aligned} \tag{4.24}$$

where the terms are for the formation of HSP·DP complexes and its dissociation, the DP refolding, the DP-dependent dissociation of an HSP·HSF1 complex and its reverse reaction.

Under physiological conditions, most of the HSF1 exists in cells in an inactive form and binds to HSP to form the HSP·HSF1 complex. Following the conjugation of HSP and DP, HSF1 is released and activated. Upon activation, HSF1 trimerizes to form $HSF1_3$ as indicated in Block 2 of Figure 4.1. The concentration of free HSF1 is obtained by the formation of the HSF1 trimer and its dissociation, the binding of HSP to DP and the release of the HSF1 monomer and its reverse process

$$\begin{aligned}\frac{\mathrm{d}[HSF1]}{\mathrm{d}t} =& -k_{2f}[HSP][HSF1] + k_{2r}[HSP\cdot HSF1] - 3k_{3f}[HSF1]^3 \\ &+ 2k_{3r}[HSP][HSF1_3] + k_{6f}[HSP\cdot HSF1][DP] \\ &- k_{6r}[HSP\cdot HSF1][HSF1]\end{aligned} \tag{4.25}$$

and the dynamics of the HSP·HSF1 complexes is given by

$$\begin{aligned}\frac{\mathrm{d}[HSP\cdot HSF1]}{\mathrm{d}t} =& k_{2f}[HSP][HSF1] - k_{2r}[HSP\cdot HSF1] \\ &- k_{6f}[HSP\cdot HSF1][DP] + k_{6r}[HSP\cdot DP][HSF1] \\ &+ k_{3r}[HSP][HSF1_3]\end{aligned} \tag{4.26}$$

where the terms are responsible for the formation of HSP·HSF1 complex and its dissociation, the DP-dependent dissociation of an HSP·HSF1 complex and its reverse reaction, and dissociation of the HSF1 trimers. In the equation governing the dynamics of the HSF1 trimer form we have terms responsible for: HSF1 trimer formation and its HSP-dependent dissociation, the SUMOylation of active trimmers $HSF1_3$ via the E2 enzyme, and the deSUMOylation of $HSF1_{3.s3}$ via the SENP

$$\begin{aligned}\frac{\mathrm{d}[HSF1_3]}{\mathrm{d}t} =& k_{3f}[HSF1]^3 - k_{3r}[HSP][HSF1_3] - k_{10}[sumo\cdot E2][HSF1_3] \\ &+ k_{11cat}[SENP\cdot HSF1_{3.s3}]\end{aligned} \tag{4.27}$$

Note that the process of phosphorylation of $HSF1_3$ is represented in Block 3 of Figure 4.1; the phosphorylation could change the function of proteins and it is essential for the

biological process. Following [4], it is assumed that the phosphatase is an excess amount compared to HSF1 and therefore the process of kinase-dependent phosphorylation in the model is omitted. The dynamic equation describing the SUMOylated $HSF1_{3.s3}$ is given by

$$\begin{aligned}\frac{\mathrm{d}[HSF1_{3.s3}]}{\mathrm{d}t} =& k_{10}[sumo \cdot E2][HSF1_3] - k_{11f}[SENP][HSF1_{3.s3}] \\ &+ k_{11r}[SENP \cdot HSF1_{3.s3}] - k_{5f}[HSF1_{3.s3}][HSE] \\ &+ k_{5r}[HSF1_{3.s3} \cdot HSE]\end{aligned} \tag{4.28}$$

The equation describing the dynamics of free HSE consists of the terms describing the bindings of $HSF1_{3.s3}$ to HSE and dissociation of those trimers from HSE in Block 5 of Figure 4.1

$$\frac{\mathrm{d}[HSE]}{\mathrm{d}t} = -k_{5f}[HSF1_{3.s3}][HSE] + k_{5r}[HSF1_{3.s3} \cdot HSE] \tag{4.29}$$

while the equation describing the dynamics of bound HSE is given by the same terms but with the opposite signs

$$\frac{\mathrm{d}[HSF1_{3.s3}] \cdot [HSE]}{\mathrm{d}t} = k_{5f}[HSF1_{3.s3}][HSE] - k_{5r}[HSF1_{3.s3} \cdot HSE] \tag{4.30}$$

The dynamics of HSP·mRNA is governed simply by the transcription and translation of HSP

$$\frac{\mathrm{d}[HSP \cdot mRNA]}{\mathrm{d}t} = k_t[HSF1_{3.s3} \cdot HSE] - k_m[mRNA] \tag{4.31}$$

Block 6 of Figure 4.1 describes the dynamics of free HSP. The binding of HSP to the DP is described by the term $[HSP][DP]$, while the dissociation of this combination is described by the term: $[HSP{\cdot}DP]$. The binding of HSP and HSF1 is described by the term $[HSP][HSF1]$ and the dissociation of this combination is described by $[HSP{\cdot}HSF1]$. The loss of the free HSP occurring due to the dissociation of HSF1 trimers and a subsequent binding of one of the HSF1 monomers to the HSP is described by the term $[HSP][HSF1_3]$. The term $[mRNA]$ models the synthesis of new HSP (for simplicity it is assumed that it is proportional to the concentration of HSP·mRNA). The terms $k_{ref}[HSP{\cdot}DP]$ and $[HSP]$

model substrate refolding and protein degradation, respectively.

$$\begin{aligned}\frac{\mathrm{d}[HSP]}{\mathrm{d}t} =& k_{mh}[mRNA] - k_{2f}[HSP][HSF1] + k_{2r}[HSP \cdot HSF1] \\ & - k_{1f}[HSP][DP] + k_{1r}[HSP \cdot DP] - k_{3f}[HSP][HSF1_3] \\ & + k_{ref}[HSP \cdot DP] - d_2[HSP]\end{aligned} \tag{4.32}$$

4.2.3 The Mathematical Model of SUMOylation of the phosphorylated HSF1 Trimer

In our heat shock model, the phosphorylated trimer $HSF1_3$ is the target protein and modified by sumo proteins. The effect of enzyme E3 is not considered in the HSR model. pre-SUMO, an immature precursor form of SUMO translation, has to be processed. pre-SUMO is transformed into a mature form in the first phase by SENP's C-terminal hydrolase activity, which cleaves the protein to reveal its diglycine residues. According to the above reactions, the dynamic of pre-SUMO is given as follows

$$\frac{\mathrm{d}[presumo]}{\mathrm{d}t} = k_{7f}[presumo][SENP] + k_{7r}[presumo \cdot SENP] \tag{4.33}$$

where the terms are for the association of SENP and pre-SUMO and its reverse reaction. The SENP does not only play a role in converting pre-SUMO into mature SUMO protein, but is also related to the deSUMOylation process. The dynamic of the free SENP is given by

$$\begin{aligned}\frac{\mathrm{d}[SENP]}{\mathrm{d}t} =& k_{7f}[presumo][SENP] + k_{7r}[presumo \cdot SENP] \\ & + k_{7cat}[presumo \cdot SENP] - k_{11f}[SENP][HSF1_{3.s3}] \\ & + k_{11r}[SENP \cdot HSF1_{3.s3}] + k_{11cat}[SENP \cdot HSF1_{3.s3}]\end{aligned} \tag{4.34}$$

where the terms are responsible for the association of SENP and pre-SUMO and its reverse reaction, and the deSUMOylation of $HSF1_{3.p3.s3}$ through binding to SENP and its reverse process. The presumo· SENP is an intermediate product; it exists briefly and then immediately changes into another form

$$\begin{aligned}\frac{\mathrm{d}[presumo \cdot SENP]}{\mathrm{d}t} =& k_{7f}[presumo][SENP] - k_{7r}[presumo \cdot SENP] \\ & - k_{7cat}[presumo \cdot SENP]\end{aligned} \tag{4.35}$$

The dynamic equation of the matured SUMO proteins is given by

$$\begin{aligned}\frac{\mathrm{d}[sumo]}{\mathrm{d}t} =& k_{7cat}[presumo \cdot SENP] - k_8[sumo][E1] \\ &+ k_{11cat}[SENP \cdot HSF1_{3.s3}] + F(T)\end{aligned} \tag{4.36}$$

where the terms are responsible for the pre-SUMO matured by SENP, the binding of sumo·E1 complex, the deSUMOylation of $HSF1_{3.s3}$ to release the free sumo protein and the target $HSF1_3$, and the direct influence from temperature change. There is some experimental evidence [36] showing that in response to heat shock, SUMO is redistributed among a wide range of proteins involved in cell cycle regulation; apoptosis; the trafficking, folding, and degradation of proteins; transcription; translation; and DNA replication, recombination, and repair. In the model, this is taken into account.

In the conjugation process, the next step is that the matured SUMO is activated by enzyme E1 and forms the complex of sumo·E1. The concentration of E1 activates sumo in an ATP-dependent manner. In the following equation, the association of sumo·E1 complex, and the binding of sumo and E2 while releasing E1 are given

$$\frac{\mathrm{d}[E1]}{\mathrm{d}t} = -k_8[sumo][E1] + k_9[sumo \cdot E1][E2] \tag{4.37}$$

and

$$\frac{\mathrm{d}[sumo \cdot E1]}{\mathrm{d}t} = k_8[sumo][E1] - k_9[sumo \cdot E1][E2] \tag{4.38}$$

Next, the conjugation of SUMO and target $HSF1_3$ occurs with E2 as a conjugating enzyme

$$\frac{\mathrm{d}[E2]}{\mathrm{d}t} = -k_9[sumo \cdot E1][E2] + k_{10}[sumo \cdot E2][HSF1_3] \tag{4.39}$$

$$\frac{\mathrm{d}[sumo \cdot E2]}{\mathrm{d}t} = k_9[sumo \cdot E1][E2] - k_{10}[sumo \cdot E2][HSF1_3] \tag{4.40}$$

The degradation and generation of $HSF1_3$ occur at the same time and $HSF1_3$ is also obtained from the dissociation of $HSF1_{3.s3}$, to form SENP· $HSF1_3$ complex. The deSUMOylation process is given by

$$\begin{aligned}\frac{\mathrm{d}[SENP \cdot HSF1_{3.s3}]}{\mathrm{d}t} =& k_{11f}[SENP][HSF1_{3.s3}] - k_{11r}[SENP \cdot HSF1_{3.s3}] \\ &- k_{11cat}[SENP \cdot HSF1_{3.s3}]\end{aligned} \tag{4.41}$$

4.3 Methodology

4.3.1 Model Parameters Optimization

A general challenge in modelling biological systems is the identification of system parameters because often these are not directly accessible experimentally [16][364–367]. In the study, the parameters (a total of 24) include forward and reverse reaction rates corresponding to the reactions, the temperature constants, and translation rate etc. In the Eqs. (4.21)–(4.32) and Eqs. (4.33)–(4.41) are derived in the previous section. None of these rate constants are explicitly known but still for some of them a reasonable range can be estimated from databases [368]. The parameters were manually tuned firstly to produce the qualitative behaviour of the system with the baselines and the reasonable ranges of the parameters taken from literature [3, 4][243][361]. For example, the value range of k_{7f} (rate of presumo and SENP association) and k_{7r} (rate of presumo·SENP complex dissociation) are [0, 4.7] and [0,1] respectively, according to the literature [243]. Set the initial value of k_{7f} and k_{7r} firstly and observe the simulation results at this value and then adjust the parameter values until a more reasonable result is obtained.

These parameters were then used as a starting point for a deeper investigation of the parameter space represented by the 24 rate constants. Genetic algorithm (GA) and differential evolution (DE) are examples of evolutionary computation. Both are essentially stochastic-based intelligent optimisation algorithms suitable for solving nonlinear combinatorial optimisation problems. The explore and exploitation of GA through the manipulation of genetic variation between population samples. DE, on the other hand, are carried out by differencing. DE is a population-based heuristic search algorithm that optimizes a problem by iteratively improving a candidate solution based on an evolutionary process [369, 370]. DE has the potential to be the most successful global optimization technique. The DE search method is quick and efficient in tackling numerical issues and locating the best overall answer. The difference is that the two calls have different selection, crossover and variation operators. If these operators in DE are replaced by those used in GA, then they are essentially the same. DE can be seen as a “modified GA algorithm” [371]. More specifically, in this study, the model parameters were estimated by using a DE based data-driven approach. DE can achieve better results than GA on numerical multi-objective

optimization problems in the HS model, which avoiding the whole population falling into a local optimum in the late stages of optimisation.

Such algorithms make few or no assumptions about the underlying optimization problem and can quickly explore very large design spaces. Because the DE is a data-driven algorithm, the author defined an objective function reflecting the quality of the fit by a root mean square error (RMSE) of the deviations between model simulations and data taken from literature [9][36] and the study's experiments. A DE algorithm was then applied to find a set of parameters which locally optimizes the objective function. Since the training data are relative values so in this study all the data were normalized between 0 (0%) and 1 (100%). For the DE algorithm, the DE parameters were set as follows: the population size was 10, the crossover probability was 0.7, the mutation probability was 0.08, and the mutation factor was 1. The smallest RMSE in each population was selected and the iteration was terminated when the fitness converged. In order to validate the model, experiments were then carried out to generate data to described in the Model verification section. The obtained data, which have been shown in Figure 4.2, include three time course curves under heat shock.

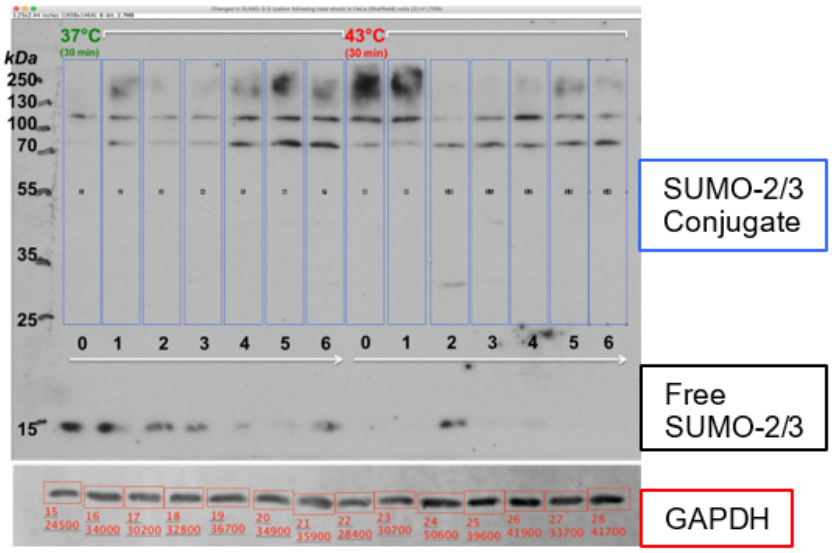

Figure 4.2. Western-blotting experiments data of SUMO conjugation

4.3.2 Model Verification by Experimental Data

HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 5mM glutamine, and 100 U/mL penicillin-streptomycin and incubated at 37 °C in humidified air with 5% CO_2 levels. Cells were pipetted into 1 mL Eppendorf tubes which were then placed into air-tight plastic bags. The cells underwent 30 min of HS treatment (43 °C) or exposure to control conditions (37 °C) in heated water baths. Afterwards, the cells were removed from the water baths and plastic bags and placed into an incubator (37 °C) to recover from HS for up to 6 h. Every hour, cell lysate samples were collected in triplicate from both the heat shock and control groups for up to 6 h of recovery time. Western blots were performed to compare relative SUMO-2/3 protein conjugation levels between experimental conditions. Cell lysate samples were prepared using a Triton X-100 lysis buffer (TLB) on ice with three additional reagents in order to detect SUMOylation: protease inhibitor at 1:50 dilution (Roche tablet EDTA-free Protease Inhibitor Cocktail 1:1000 in water); 0.1% sodium dodecyl sulphate (SDS); and 20 mM N-ethylmaleimide (NEM) added to the lysis buffer immediately before use, as previously described [28].

At the appropriate time points, the cells were removed from the incubator and spun down at 4,000 rpm for 2 min. Excess media was removed from the Eppendorf tubes and 60μL TLB was pipetted into each tube. The cells were allowed 15 min for lysis on ice. The cell lysates were then sonicated and spun down for 15min at 4 °C and 13,200 rpm, and transferred into new Eppendorf tubes. A 6 x Laemmli buffer was added to each tube. The samples were stored at -80 °C until further use. Bond Breaker^{TM} TCEP (1:10; Sigma) was added to aliquots of each cell lysate sample. The samples were spun down for 30 s at 13,200 rpm, vortexed briefly, and heated at 70 °C for 10 min. SDS-PAGE (polyacrylamide gel electrophoresis) was performed to separate protein bands. Proteins were transferred onto Immobilon®-FL PVDF membranes (Millipore). Immunoblotting was performed on the membranes to detect SUMO-2/3 levels using primary antibodies for SUMO-2/3 (Cell-Signalling, rabbit) and GAPDH (Santa Cruz, mouse).

Antibody complexes were detected with HRP-conjugated secondary antibodies (Sigma) or IRDye 800CW/680RD near-infrared fluorescent secondary antibodies (LI-COR Bioscience, Ltd.) and then developed by chemiluminescence using Luminata^{TM} Forte Western HRP

Substrate (Merck Millipore) or Amersham^{TM} ECT^{TM} Prime or Select Western Blotting Detection Reagent (GE Healthcare Life Sciences) on film (CL-XPosure^{TM} Film, Thermo Scientific^{TM}) or scanned with Li-COR Odyssey machine. ImageJ and Image Studio Lite were used for protein quantifications, respectively.

4.3.3 Global Sensitivity Analysis

Sensitivity analysis approaches have been used to analyse biological mechanisms, including metabolic networks, signalling pathways, and genetic circuits, as a result of the growing use of systems biology. Sensitivity analysis may reveal how resilient biological reactions are to changes in biological parameters, as well as which model inputs are the most important elements influencing model outputs. Furthermore, sensitivity analysis is useful for directing experimental investigation, model reduction, and parameter estimation. The two forms of sensitivity analysis widely used in biological systems are local and global sensitivity analysis methods. A typical approach for studying the influence of small disturbances on model outputs is local sensitivity analysis. Conversely, global sensitivity analysis methods have been used to determine how significant alterations in model input parameters affect model outcomes [372].

Sensitivity analysis is crucial in the dynamic study of biological mechanisms. It may reveal how resistant the model outputs are to changes in model inputs, as well as which model inputs are the most important influences on the model output. Moreover, sensitivity analysis is critical in the construction of models. It improves parameter estimates and experimental design by allowing us to verify whether a model prediction is based on model assumptions [373, 374]. By measuring the dependency of model outputs on their inputs, sensitivity analysis is also valuable for model simplification. Some procedures may be simplified or removed, and the values of insensitive parameters may be fixed [375]. Finally, sensitivity analysis can help drive experimental analysis. With sensitivity analysis, it is possible to determine which model inputs are primarily responsible for the variance in model outputs (experimental observations). Further experimental study might focus on the most sensitive model input parameters and their related biological processes [372]. Sensitivity analysis has been used in a variety of domains including environmental modelling, economic decision-making modelling, chemical kinetics, and

biological modelling analysis [376–378].

The influence of input factors (parameters) on output variables is studied using sensitivity analysis. As previously stated, local sensitivity analysis (LSA) and global sensitivity analysis (GSA) two forms of sensitivity analysis. In LSA, the effect of a small parameter disturbance on outputs is investigated one at a time, while other parameters are kept at their normal levels. On the other hand, GSA investigates the impacts of individual model parameters (within feasible ranges) and the interaction/combination impacts of all parameters on outputs variables. In this chapter, a variance-based Sobol's method is used to analyse the impact of model input parameters and their interactions on output variables in a mathematical model of HS. Variance-based sensitivity analysis is a form of global sensitivity analysis. The results of Sobol's method for global sensitivity analysis are described in section 4.4.2.

4.4 Results

4.4.1 Simulation and Experiment Results

In order to test the model, the author studied the dynamics of the system triggered by exposure to the heat shock pattern as shown in Figure 4.3, where the temperature starts at 43 °C from the beginning of the simulation and lasts 1 h then drops back to 37 °C. The entire simulation time is 7 h. Note that this pattern is in line with our experiment for the purpose of validation. The baseline values of the parameters used for the model is shown in Table 4.1.

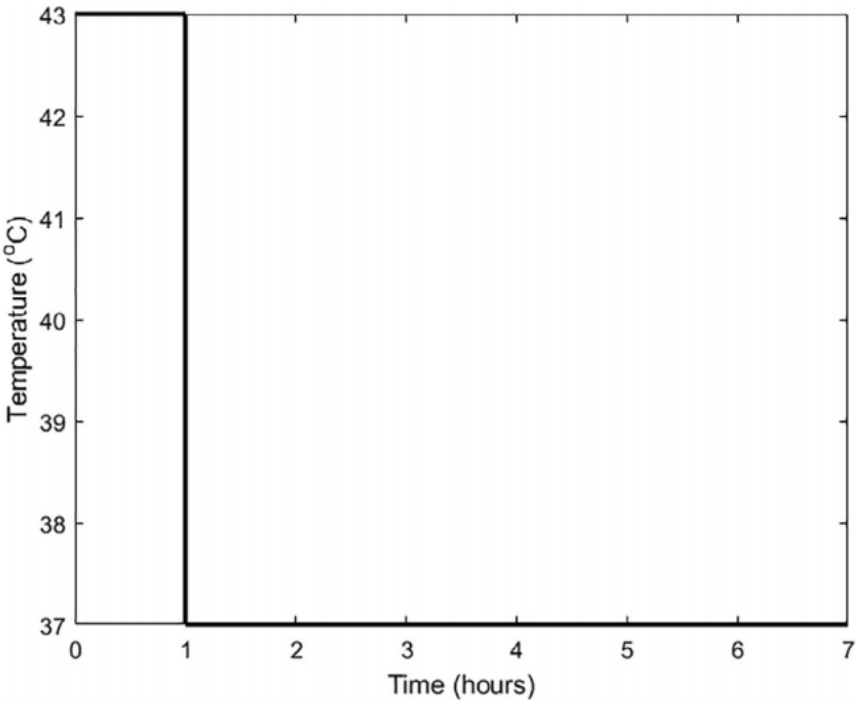

Figure 4.3. The temperature pattern for the heat shock simulation

Table 4.1. The baseline values of the parameters of the model

Parameters	Parameter Description	Values
k_{1f}	Rate of HSP and DP association	0.42 [4]
k_{1r}	Rate of HSP·DP complex dissociation	0.005 [4]
k_{2f}	Rate of HSP and HSF1 association	0.42 [4]
k_{2r}	Rate of HSP·HSF1 complex dissociation	0.005 [4]
k_{3f}	Trimers formation rate of HSF1 (HSF1 activation rate)	0.023 [4]
k_{3r}	Trimers dissociation rate of HSF1 (HSF1 inactivation rate)	0.00575 [4]
k_{mh}	Rate of HSP translation	0.4
k_m	Rate of HSP·mRNA complex degradation	0.035 [4]
k_{5f}	Rate of $HSF1_{3s3}$ and HSE association	0.035 [4]
k_{1r}	Rate of $HSF1_{3s3}$·HSE complex dissociation	0.035 [4]
k_{6f}	Rate of HSF1 dissociation and formation of HSP·DP complex	0.023 [4]
k_{6r}	Rate of DP dissociation and formation of HSP·HSF1 complex	0.00036 [4]
k_{ref}	DP refolding rate	0.014 [4]
d_2	Rate of HSP degradation	0.013 [4]
k_t	HSP transcription rate	0.035 [4]
k_{7f}	Rate of presumo and SENP association	0.72 [243]
k_{7r}	Rate of presumo·SENP complex dissociation	0.0242
k_{7cat}	Catalytic rate of presumo·SENP complex	0.75
k_8	Rate of sumo and E1 association	0.926
k_9	Rate of E1 dissociation and formation of sumo·E2 complex	0.101
k_{10}	Rate of E2 dissociation and formation of sumo $HSF1_3$ complex $HSF1_{3s3}$	0.52
k_{11f}	Rate of SENP and $HSF1_{3s3}$ association	0.1
k_{11r}	Rate of SENP·$HSF1_{3s3}$ dissociation	33.1 [243]
k_{11cat}	Catalytic rate of SENP·$HSF1_{3s3}$ complex	0.75

The simulation results of the model are displayed in Figures 4.4–4.8. Figure 4.4 shows the validation results by comparison between experimental data and the model predicted values for the level of conjugated SUMO following the single heat shock. The root mean square error (RMSE) between the predicted level and the experimental data is 0.02, which indicates a very good agreement. Given that the lasting time for heat shock is 1 h, it can be observed from Figure 4.4 that immediately upon the heat shock, the concentration of the SUMO proteins starts to rise and reaches the peak in around 1 h and 10 min, the exact time as the three experimental observations. After that the level of conjugated SUMO proteins drops gradually, which indicates that after the heat shock, SUMO proteins

gradually deconjugate from substrates to become free SUMO. The result is also consistent with the experimental results in the literature [9][36].

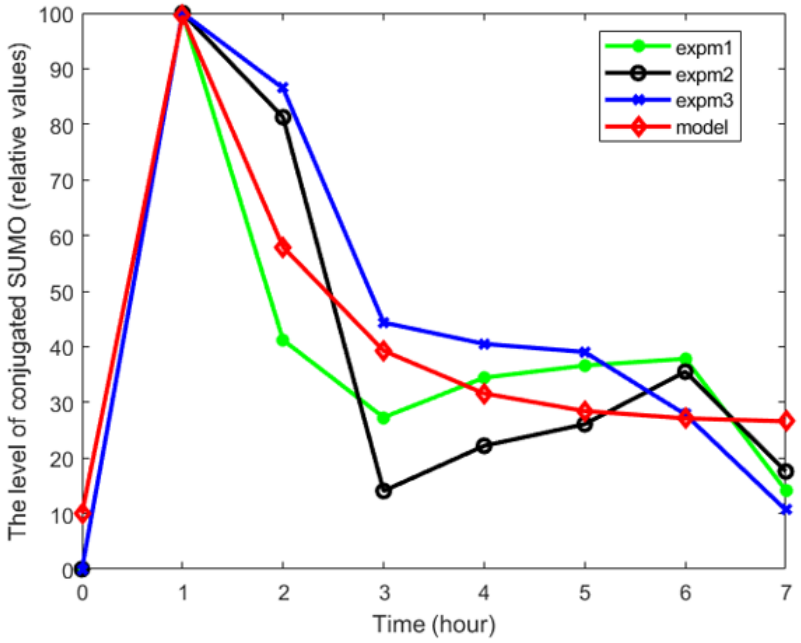

Figure 4.4. Comparison between experimental data and the model predicted values for the level of conjugated SUMO following a single heat shock

Figure 4.5 indicates the 95% confidence intervals of experiment data and model prediction data for SUMO conjugation level. A 95% confidence intervals of the mean is a range with an upper and lower number calculated from a sample. Because the actual population mean is unknown, this range describes possible values that the mean could be. Although 99% and 90% confidence intervals might also be calculated, 95% confidence intervals are the most commonly used. Thus, the author will concentrate on them in this section. Confidence intervals can be derived for various data, including odds ratios and percentages, but for simplicity, the author only considers the mean. A 95% confidence intervals of mean is [16.43447, 66.85982], [10.85102, 73.90326], [20.31445, 79.35697] and [19.87852, 69.55005] in three experimental data sets and model prediction data, respectively. The range is narrower, a more precise estimate. For example, in Figure 4.5, in comparison with the model prediction result, the confidence intervals mean range for the first experiment data is narrower than the second and third experiment data sets. It suggests that the first experimental data set is more informative than others in the HS model.

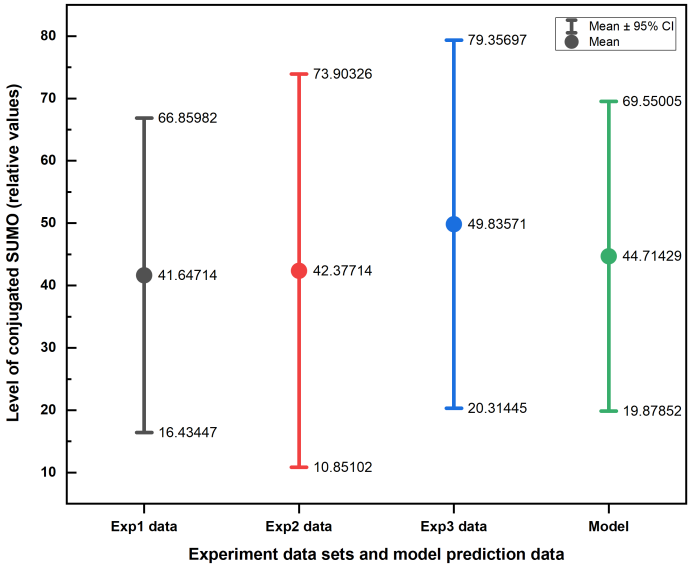

Figure 4.5. 95% confidence interval analysis for experimental data and model prediction result of SUMO conjugation level

Figures 4.6 and 4.7 show the effect of the heat shock on the denatured protein (DP); the effect of the heat shock on the protein synthesis in the cell (mRNA); and the effect of the heat shock on the HSF1 trimer in the cell. Figure 4.6 shows the rapid accumulation of the denatured protein upon the increase of the temperature, which reaches the peak around the end of the heat shock. Then the concentration of the denatured protein drastically drops to zero within about 10 min. This indicates the effect of the HSP playing a chaperone role for repairing the damaged proteins.

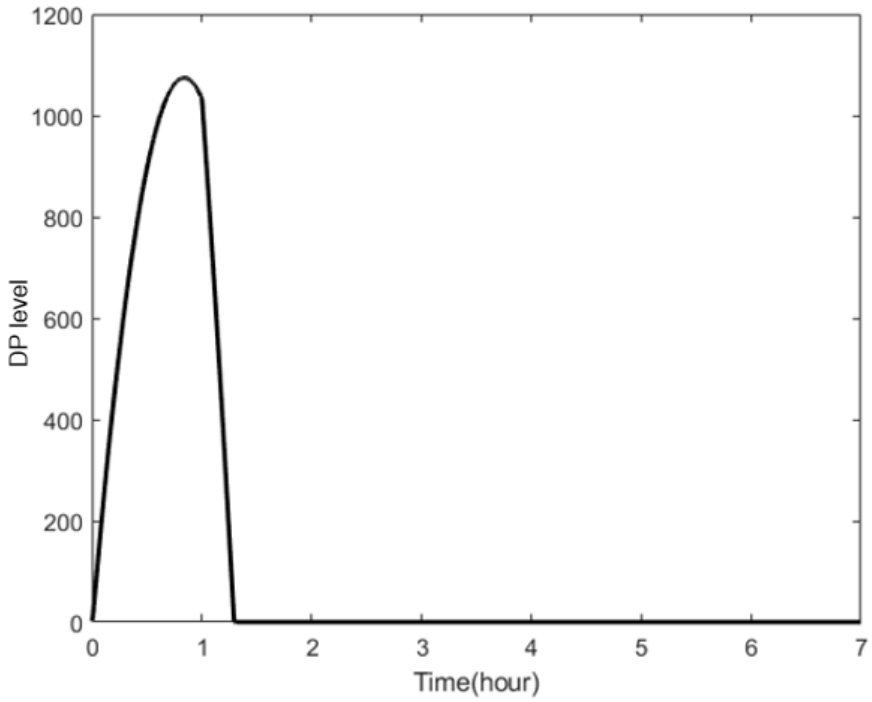

Figure 4.6. The concentration of the denatured proteins in response to the single heat shock

Figure 4.7 shows the concentration of the HSF1 trimer, which represents the rapid increase of the binding of the HSF1s for the purpose of the HSP synthesis. These results are consistent with the experimental results and simulation results in the literature [4][12][36].

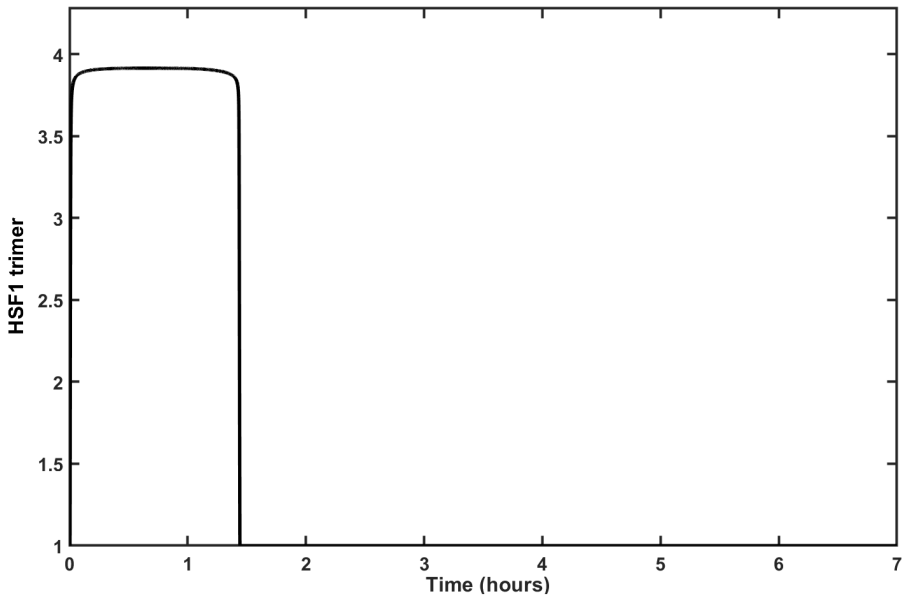

Figure 4.7. The concentration of the HSF1 trimer in response to the single heat shock

Figure 4.8 expresses the dynamics of the conjugated SUMO proteins resulting from computational simulations of Eqs. (4.16)–(4.27) where the temperature varies from 37°C

up to 40 °C (dotted line), 41 °C (dashed line) and 42 °C (solid line). From these simulations it can be observed that the level of the conjugated SUMO proteins follows the well-known profile of an initial rapid increase followed by a slower attenuation and finally a return to a value close to its physiological level.

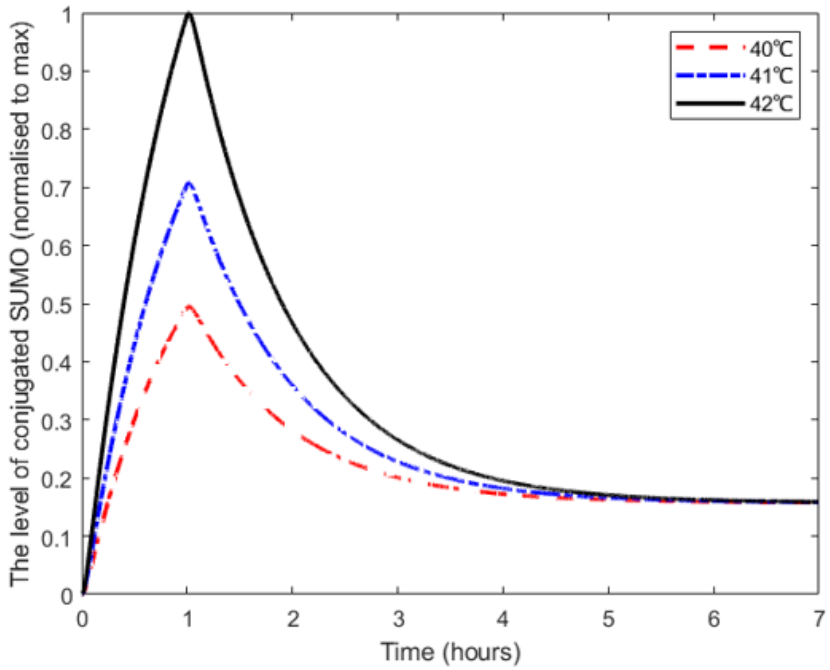

Figure 4.8. Different profiles of the conjugated SUMO proteins obtained for the temperature signals corresponding to heat shock from 40 °C (dotted line), 41 °C (dashed line) and 42 °C (solid line)

Figure 4.9 represents the simulation result of the conjugated SUMO proteins following two successive heat shocks of 42 °C. In this simulation, the temperature has a sudden rise to 42 °C at hour 0 lasting 30 mins and returns to 37 °C for the first heat shock; the temperature has a sudden rise to 42 °C at hour 4 lasting 30 mins for and then returns to 37 °C for the second heat shock. The figure demonstrates that the second heat shock is substantially less than the effect of the first one, probably due to a slow deSUMOylation process.

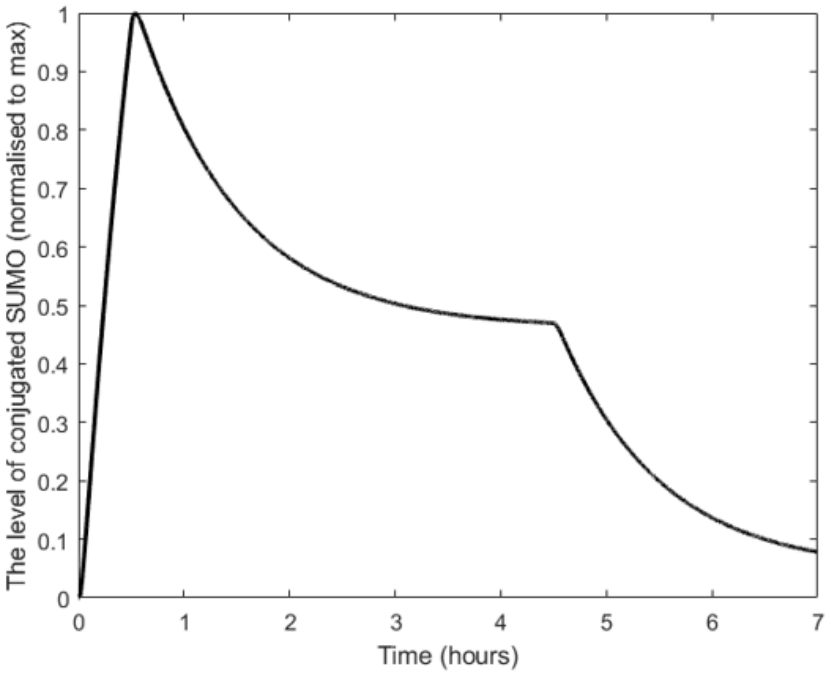

Figure 4.9. The level of conjugated SUMO following the double heat shock

Figures 4.10–4.12 show the simulated concentration of HSF1, mRNA and HSP70, respectively. It can be observed that in response to the heat shock, the level of HSF1 increases rapidly due to the protein synthesis in Figure 4.10 before which there is a period when the level of HSF1 is strongly decreased due to its trimerisation (compared with Figure 4.7).

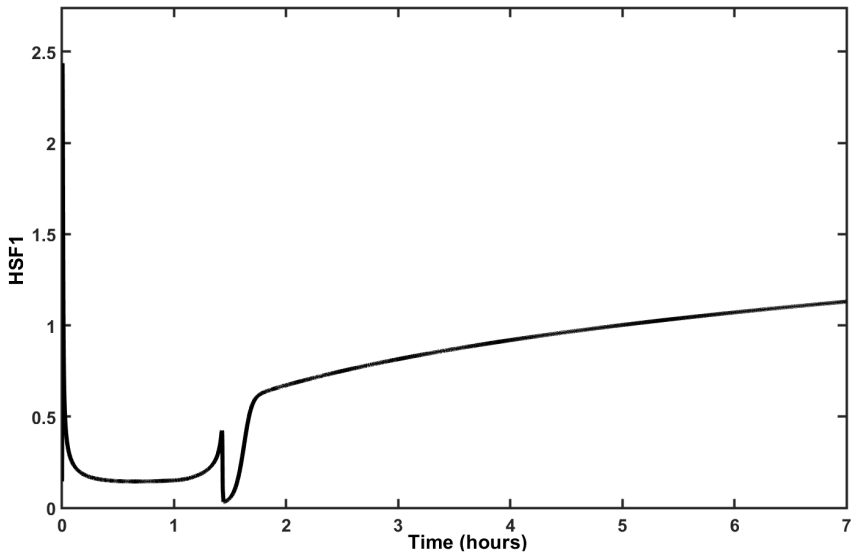

Figure 4.10. The simulated concentration of the HSF1 in response to the single heat shock at 43 °C

Figure 4.11 indicates the predicted dynamics of HSP70 and mRNA, where there is a rapid increase in HSP70·mRNA synthesis, followed by a return to its physiological level.

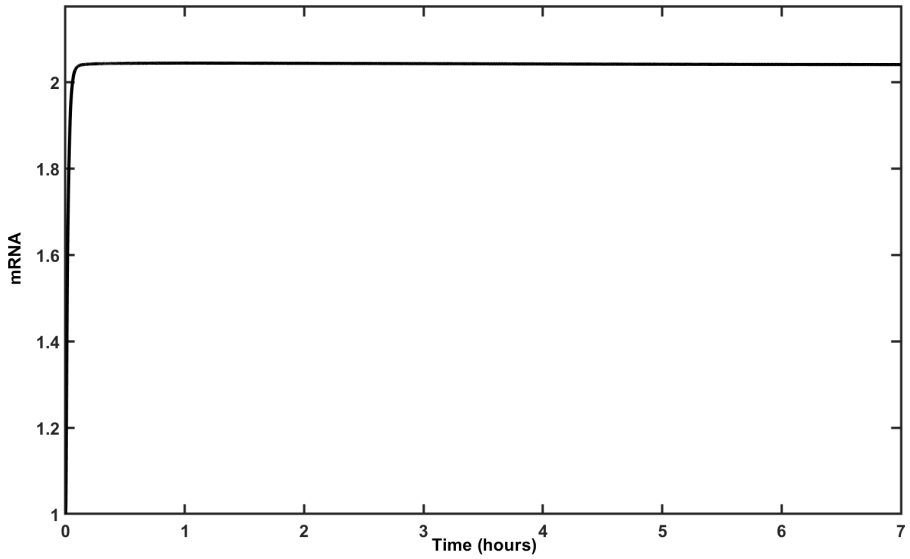

Figure 4.11. The simulated concentration of the mRNA in response to the single heat shock at 43 °C

For HSP70, it can be observed in Figure 4.12 that there is an increase in the level of HSP70 in the cell after the heat shock and a decrease in the level of HSP70 bound to denatured proteins. These results are qualitatively consistent with the results in the literature [4][15][361].

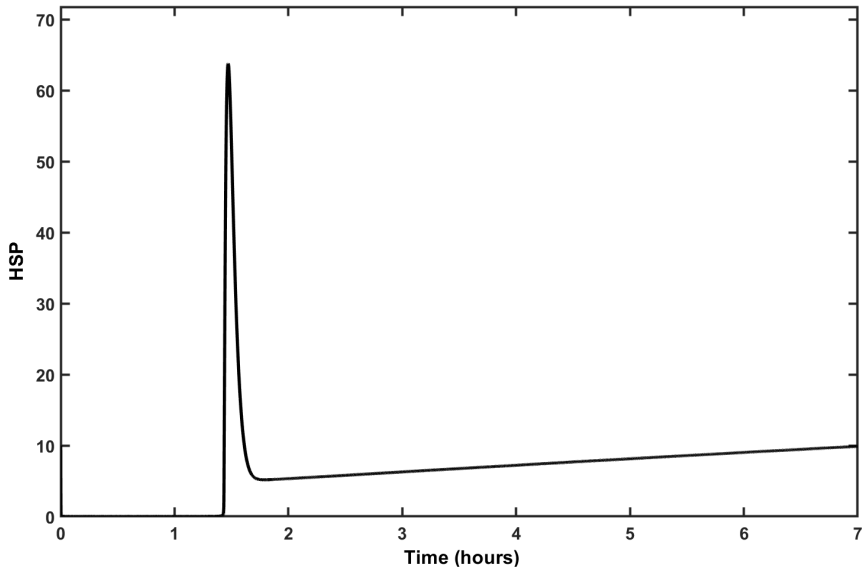

Figure 4.12. The simulated concentration of the HSP in response to the single heat shock at 43 °C

In summary, the level of the free HSP decreases after a single heat shock when it binds to denatured proteins in the model. However, a drop in the amount of HSP very instantly causes a complete activation of the available HSF1 in the cell due to the nonlinear behaviour of the trimerization process of HSF1 and its short time constant. Therefore, double heat shock was applied to represent better the relationship between SUMO conjugation levels and heat shock stress. The constitutive level for the first heat shock is the SUMO conjugation level. The level of conjugated SUMO had substantially grown when the second heat shock was delivered. In addition, the level of conjugated SUMO under different temperature signals is also described. The results show that the conjugated SUMO is responsive to different temperatures, suggesting a strong association between SUMO conjugation level and heat shock.

4.4.2 GSA Computational Results for HS Model

The Sobol's indices as the variance-based sensitivity analysis is a form of global sensitivity analysis. The S_i is also known as the "main effect indices" or "first-order sensitivity indices", While the S_{Ti} is called the "total-order indices" or "total effect indices". The significance of each variable in influencing the output variance may be seen using the S_i, S_{Ti}. The GSA may provide insightful information about the robustness of biological responses to changes in biological parameters and which model inputs are most important in influencing the model outputs [372]. The results of both sensitivity indices are presented in Figure 4.13-Figure 4.31 for N=5000 model runs per parameter.

Figure 4.13 shows that the parameter k_{1f} has both a strong main effect and interaction effect on DP, while k_{1r} and k_{2f} have smaller effects on DP than k_{1f}.

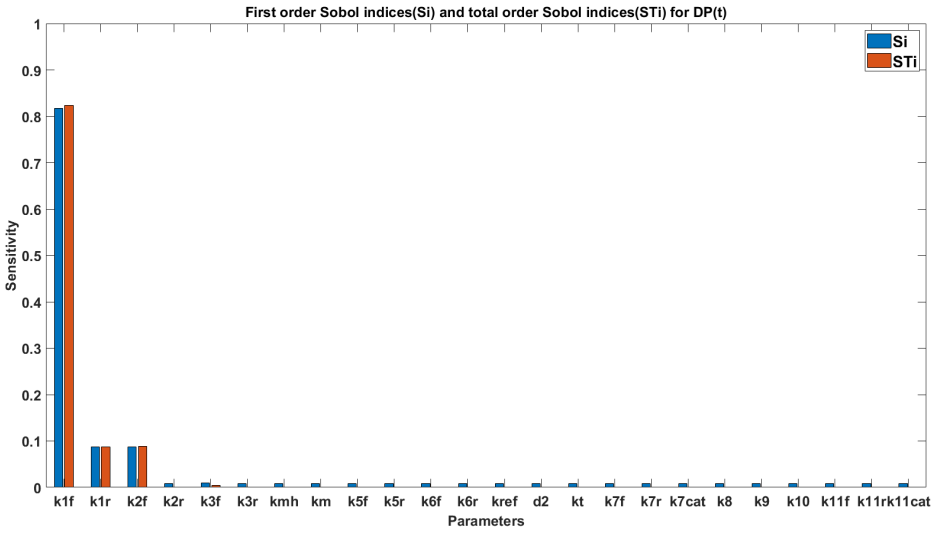

Figure 4.13. First- and Total-order of Sobol's Indices for DP(t)

The parameters k_{1f}, k_{mh}, k_{2f} and k_{7cat} indicated major contributions of main and interaction effects in output for HSP as shown in Figure 4.14.

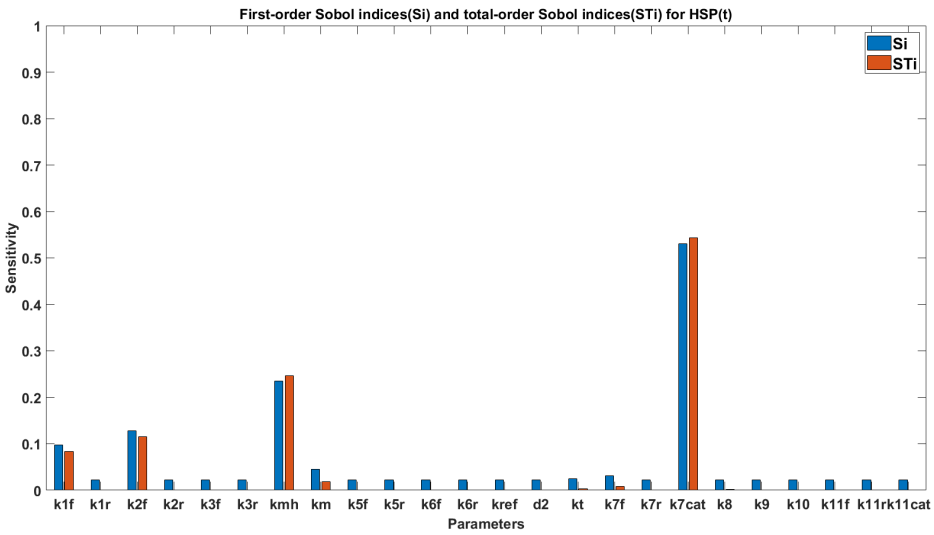

Figure 4.14. First- and Total-order of Sobol's Indices for HSP(t)

Figure 4.15 demonstrates that k_{1f}, k_{2f} and k_{ref} are most influential on both main and interaction effects than on other parameters for the HSP·DP complex.

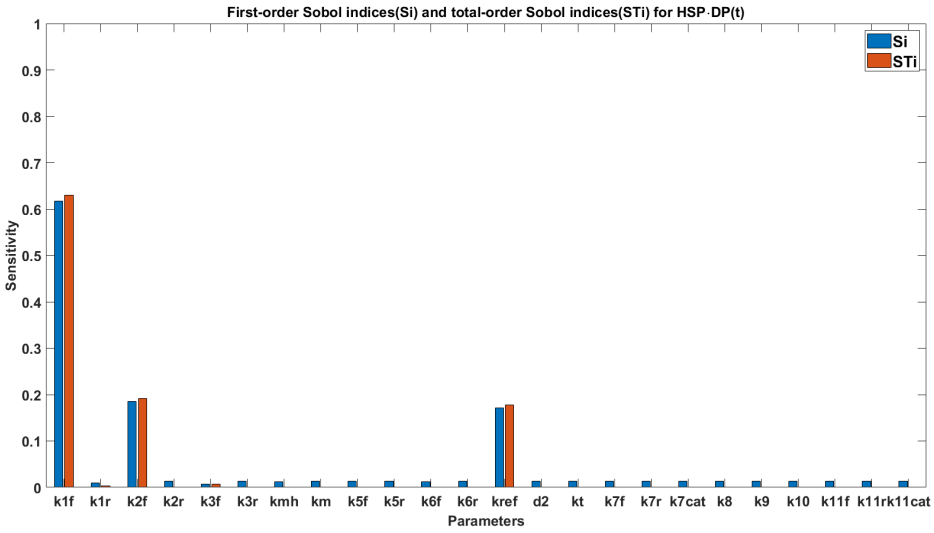

Figure 4.15. First- and Total-order of Sobol's Indices for HSP·DP(t)

The parameter k_{2f} displayed strong main and interaction impacts in output only for both HSF1(t) and HSP·HSF1(t) in Figure 4.16 and Figure 4.17, respectively.

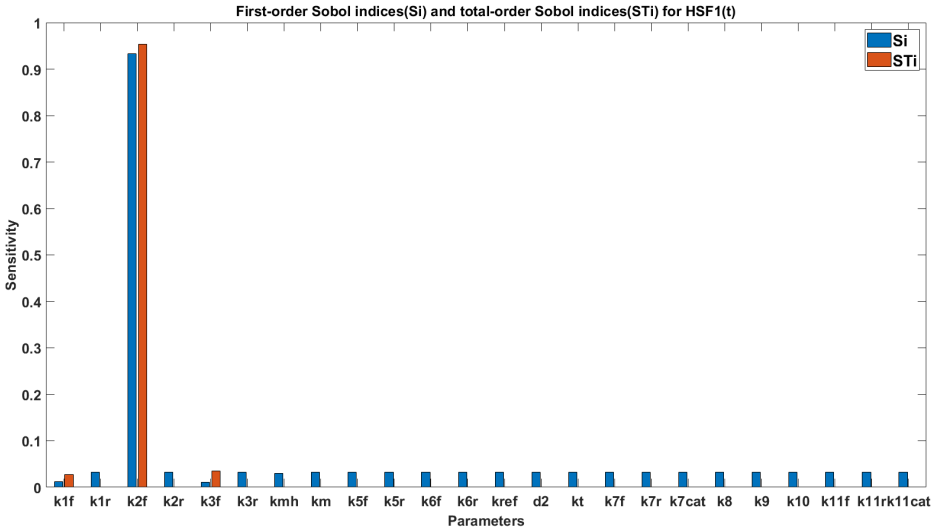

Figure 4.16. First- and Total-order of Sobol's Indices for HSF1(t)

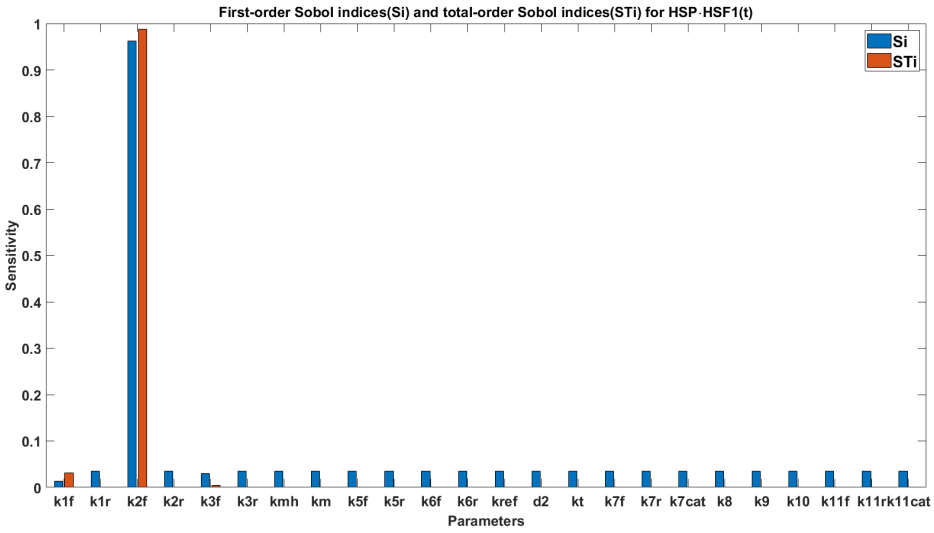

Figure 4.17. First- and Total-order of Sobol's Indices for HSP·HSF1(t)

The main and interaction effects of k_m and k_{7cat} in output uncertainty for mRNA(t) are shown in Figure 4.18.

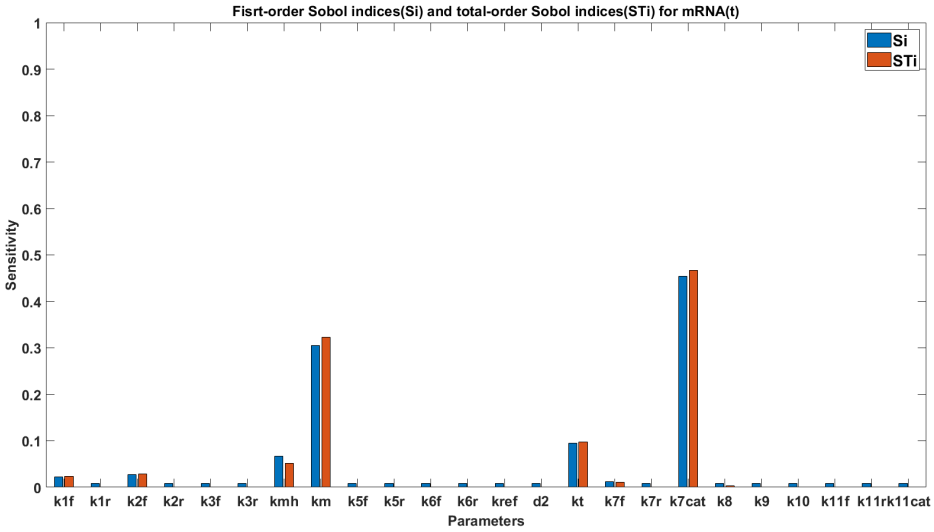

Figure 4.18. First- and Total-order of Sobol's Indices for mRNA(t)

Figure 4.19 shows that parameters k_{3f} and k_{7cat} have large main and interaction effects on $HSF1_3$(t).

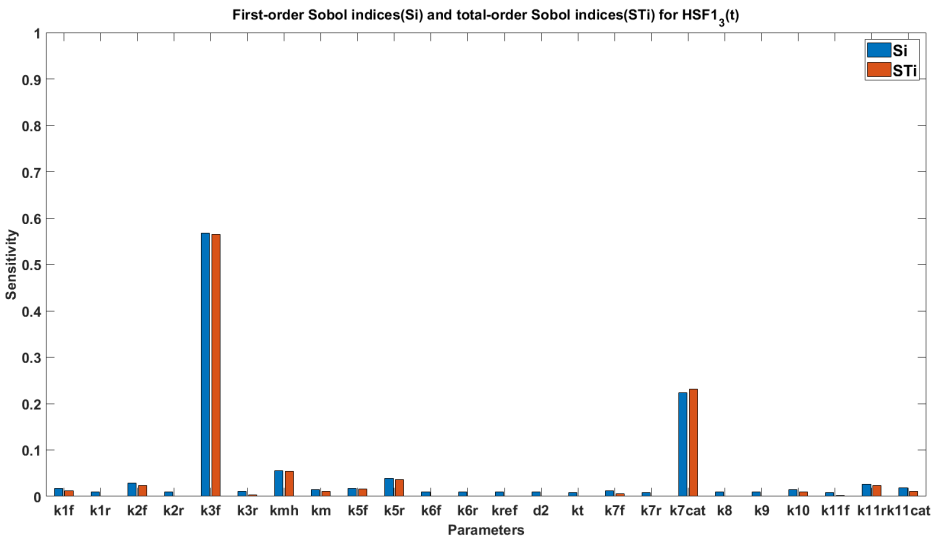

Figure 4.19. First- and Total-order of Sobol's Indices for HSF1$_3$(t)

The parameter k$_{7cat}$ indicated major contributions in output uncertainty for both HSF1$_{3s3}$(t) and presumo(t) as shown in Figures 4.20-4.31, respectively.

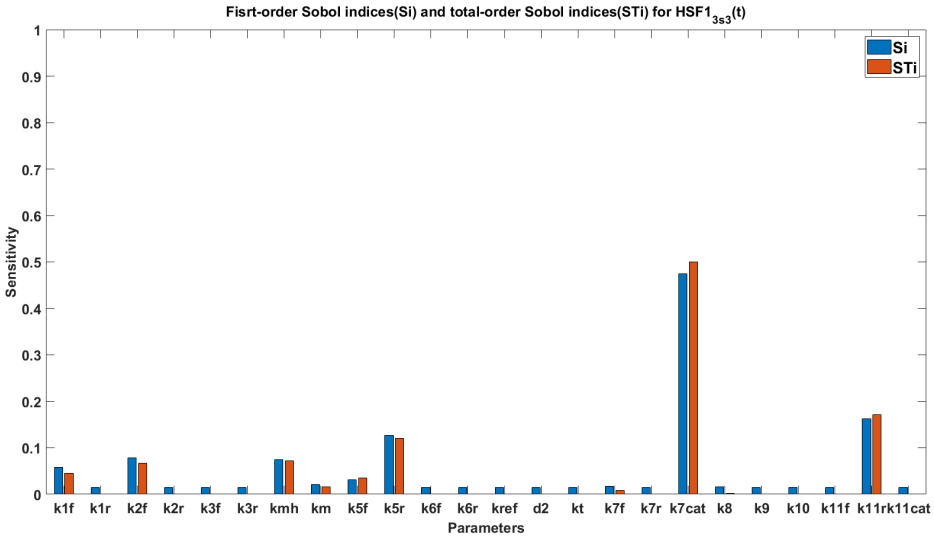

Figure 4.20. First- and Total-order of Sobol's Indices for HSF1$_{3s3}$(t)

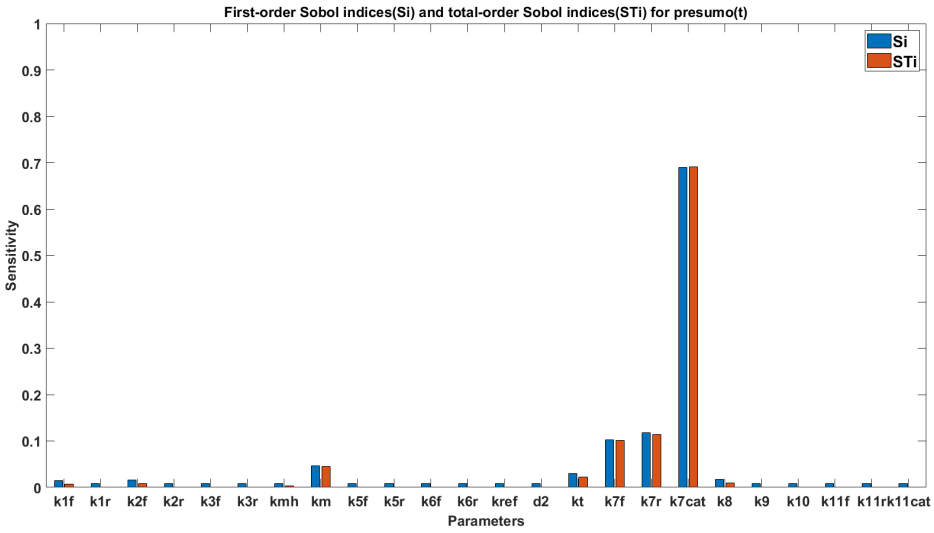

Figure 4.21. First- and Total-order of Sobol's Indices for presumo(t)

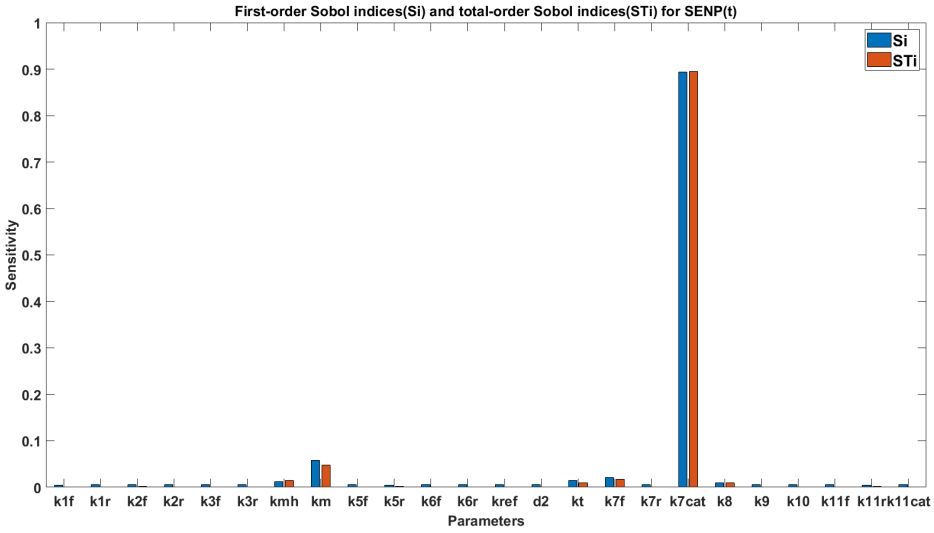

Figure 4.22. First- and Total-order of Sobol's Indices for SENP(t)

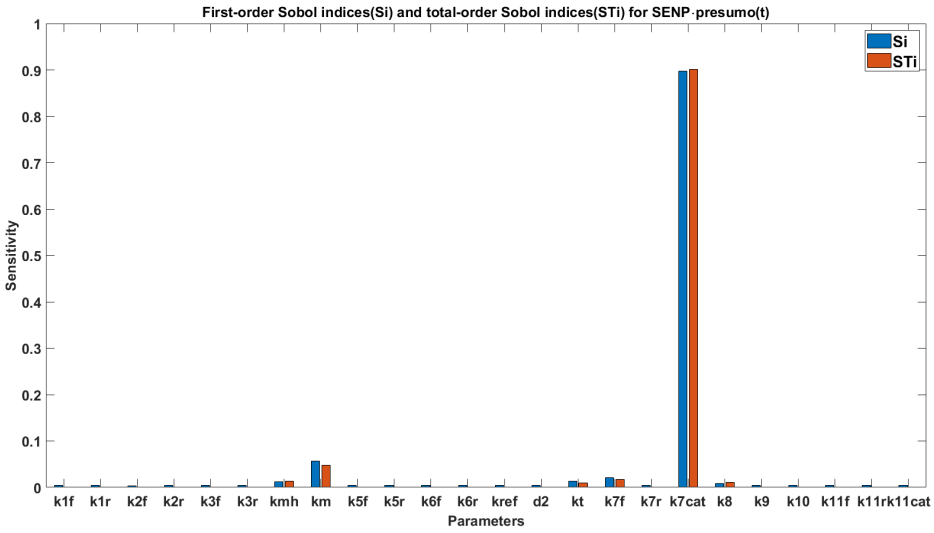

Figure 4.23. First- and Total-order of Sobol's Indices for SENP·presumo(t)

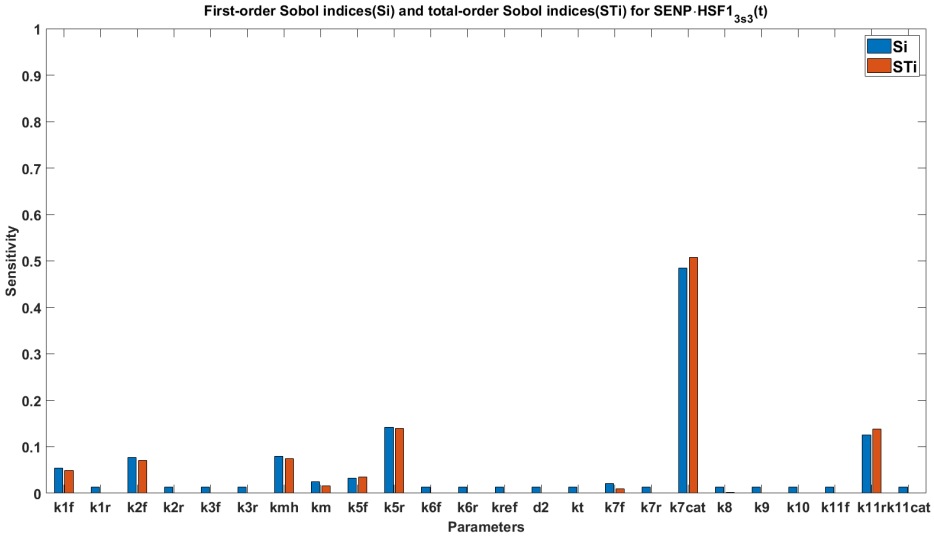

Figure 4.24. First- and Total-order of Sobol's Indices for SENP·HSF1$_{3s3}$

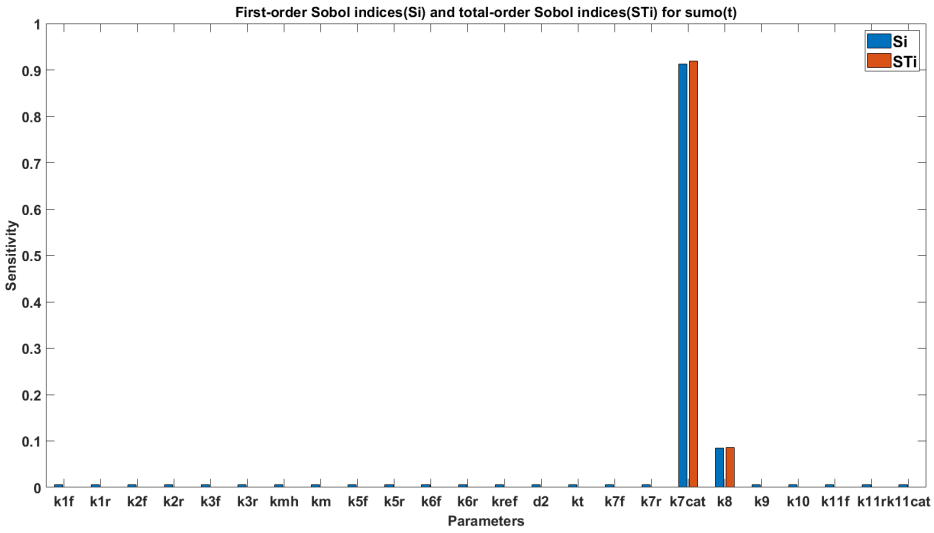

Figure 4.25. First- and Total-order of Sobol's Indices for sumo

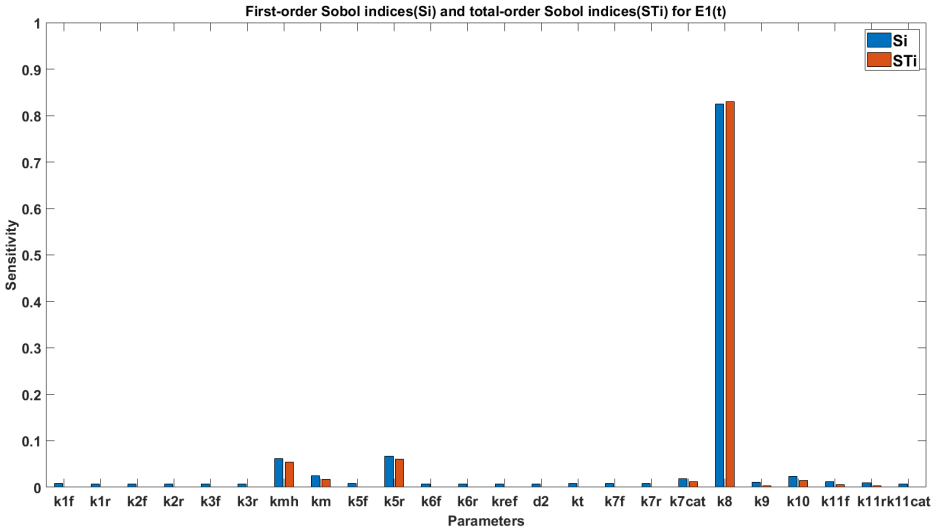

Figure 4.26. First- and Total-order of Sobol's Indices for E1

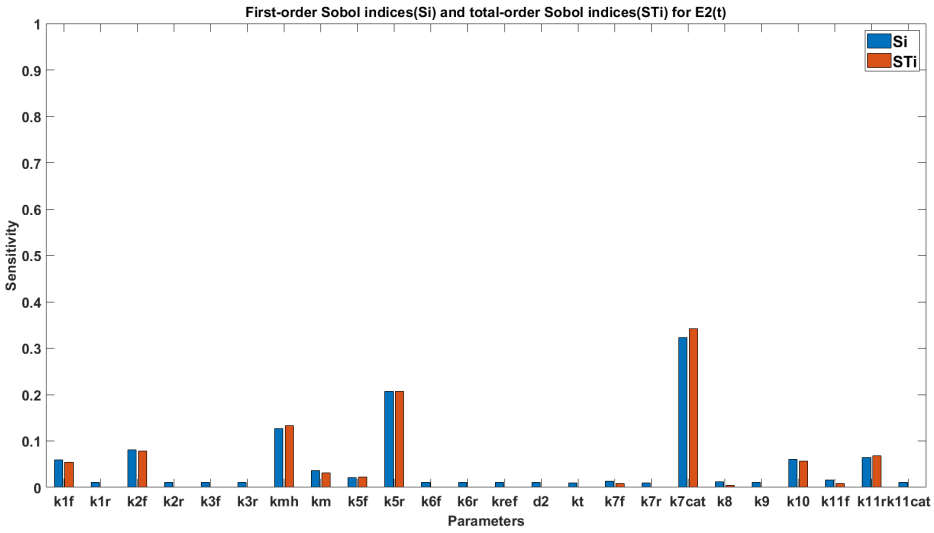

Figure 4.27. First- and Total-order of Sobol's Indices for E2

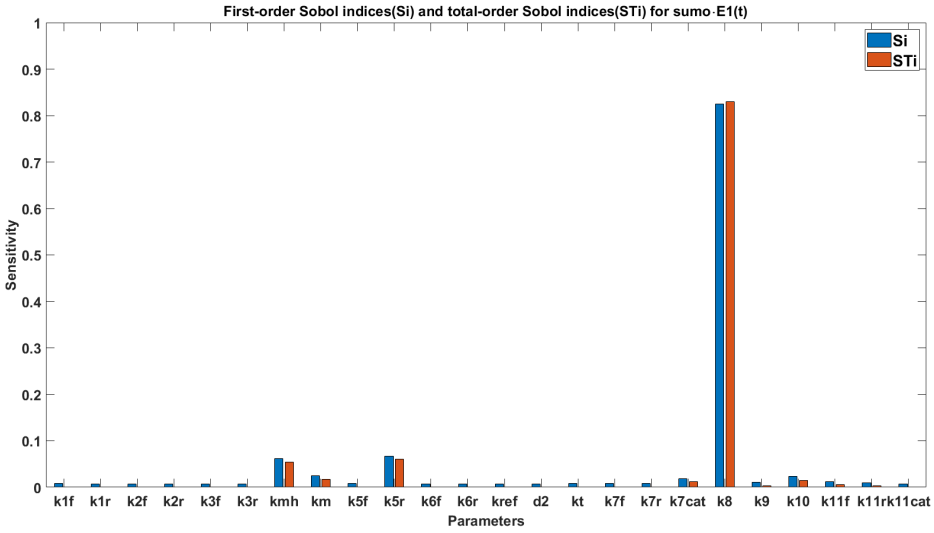

Figure 4.28. First- and Total-order of Sobol's Indices for sumo·E1

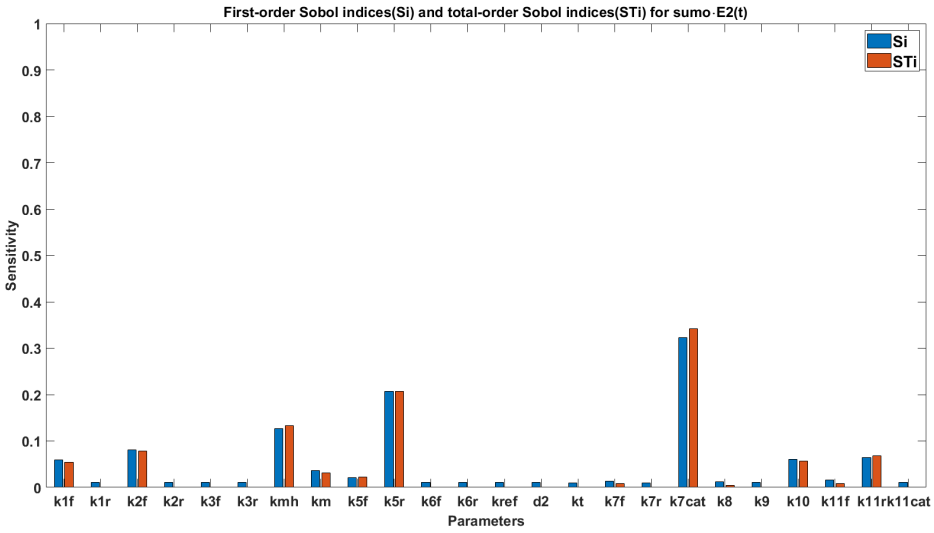

Figure 4.29. First- and Total-order of Sobol's Indices for sumo·E2

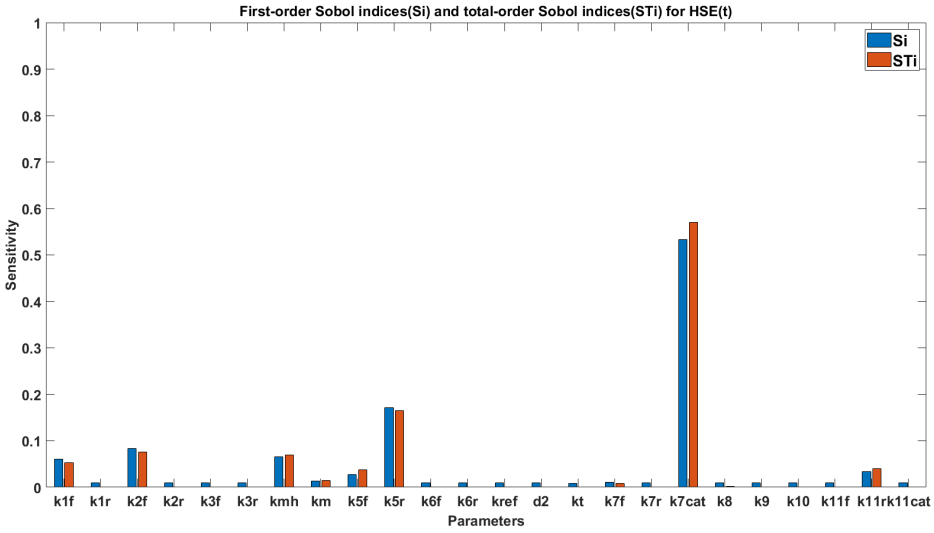

Figure 4.30. First- and Total-order of Sobol's Indices for HSE

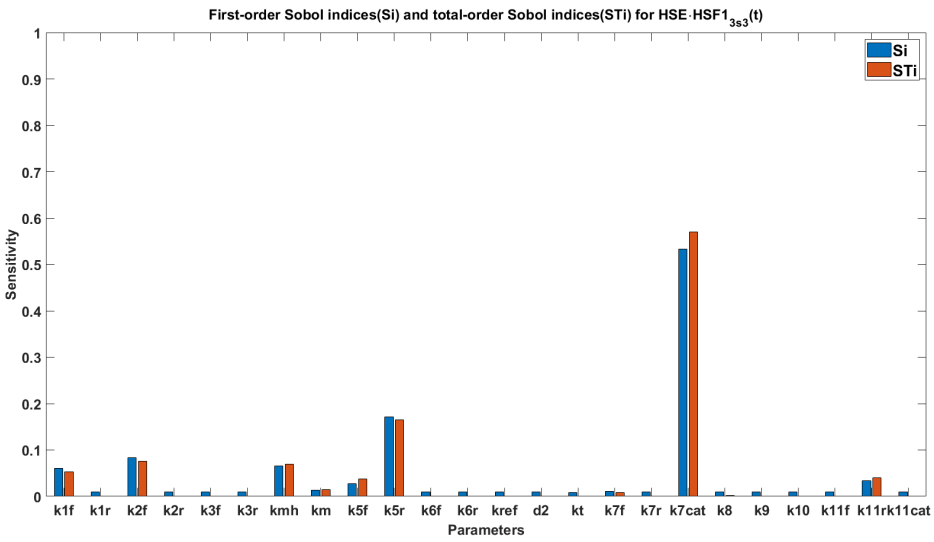

Figure 4.31. First- and Total-order of Sobol's Indices for HSE·HSF1$_{3s3}$

It can be observed from GSA results that the input parameters k_{7cat} and k_8 have the most critical influence on the output variables that are involved in the SUMOylation mechanism; the k_{1f} and k_{2f} influences proteins to refold process. The sensitivity results investigate a combination of biological and mathematical experiments. Based on the parameters of variation, the found sensitive parameters, and the stability of the healthy steady state, the dominating parameters for each experiment may be recognised.

4.5 Conclusion

Since SUMO was discovered to be a reversible post-translational protein modifier in the 1990s [379], many enzymes that participate in regulated SUMO-conjugation and -deconjugation pathways have been identified and characterized [219][380]. It has been found that SUMOylation plays an essential role in cellular processes and its outcomes are extremely diverse, ranging from changes in localisation to altered activity and, in some cases, stability of the modified protein [34][211][216]. Regarding HSR, it has been observed that HSF1 is very rapidly and transiently SUMOylated upon heat stress [296]. In recent years, experimental studies have provided strong evidence that SUMOylation, as a PTM, is a key determinant of cell fate in response to extreme stresses, including heat shock stress [28, 29][36]. It has been noticed that the SUMO system has the ability to not only rapidly respond to hyperthermic stress, but also to have a broad range of signal durations,

which suggests a role for SUMO in both short-term damage repair and long-term protection from future insult, which is characteristic of the heat shock response [381]. However, how SUMO exerts its function in transcription regulation is largely unknown [29].

In this chapter a mathematical modelling in combination with an experimental validation for studying cell response to HS have been presented. The author have focussed on the mechanism of SUMOylation of the heat shock transcription factors (HSF1s) in the biological process. The research on cell HSR using mathematical models has been conducted extensively over the past decades [3, 4][15, 16]. In [15], a heat shock model proposed by Peper *et al.* for predicting the cellular response to both a single and two consecutive HSs appears to closely resemble experimental data on HSP70 synthesis. It has been proposed that the higher the temperature (in the range of 37 °C to 43 °C), the higher the concentration of HSP synthesis [4]. However, the role of SUMOylation in the heat shock response has never been studied by using mathematical models. In this thesis, to the best of my knowledge, for the first time, the mechanism of SUMOylation in heat shock response is studied by using mathematical modelling techniques.

In this chapter, using the law and mass action and Michaelis-Menten kinetics, the biochemical rate reactions were converted into a system of ordinary differential equations (ODEs) which described the underlying biological process. Global sensitivity analysis (GSA) is used to display the influence of input factors on output variables of the HS model. This makes it intuitively simple to obtain output results by adjusting the input parameters. Furthermore, in order to validate the model, time-course data of the heat shock response of the cell ware generated by our experiments. Because the author focused on the role of SUMOylation, the recorded data were the relative level of conjugated SUMO proteins which are in very good agreement with the experimental data (RMSE 0.02). The direct influence from temperature change on the dynamics of the SUMO proteins implies the potential role of the heat shock in the recruitment of SUMO proteins to participate in the entire cellular recovery programme. This is supported by some experimental evidence [36] where, in response to HS, SUMO is redistributed among a wide range of proteins involved in multiple cellular events: cell cycle regulation; apoptosis; intracellular trafficking, folding, and degradation of proteins; transcription; translation; DNA replication, recombination, and repair.

The developed model has been employed in simulating interesting conditions that have

not yet been tested experimentally. In the first scenario, the developed model was used to predict the outcome of the conjugated SUMO proteins when a cell was exposed to three different temperatures, 40 °C, 41 °C, and 42 °C, respectively. The HS had a sudden increase of temperature at the start of the simulation, which lasted 1 h and then dropped back to 37 °C. The computational results of the model showed that the higher the temperature is, the higher the level of the conjugated SUMO proteins, which indicates the direct link between temperature and the SUMOylation level. In the second scenario, the model was used to predict the effect of two successive heat shocks of 42 °C: the temperature has an unexpected rise to 42 °C at hour 0, which lasts for 30 mins and returns to 37 °C for the first HS; the temperature has a rapid rise to 42 °C at hour 4, which lasts for 30 mins and then returns to 37 °C for the second HS. The figure shows that the effect for the second HS is substantially less than for the first one, probably due to the influence of de-SUMOylation process in the first HS on the second HS. Overall, the computational simulation results have demonstrated that cells can adapt to and survive the application of a moderate HS. Also the author has demonstrated the predictive power of the model and its usefulness in providing new insights of the system's dynamics.

SUMOylation triggered by cellular stresses such as heat shock stress could be a fundamental molecular mechanism for cells in response to various stimuli to survive. Current evidence illustrates that increased SUMO-2/3 conjugation following HS is essential for cell survival [35, 36]. In addition, experimental studies conducted in mice have revealed that the accumulation of misfolded proteins, most likely following dysfunctional cell stress responses, are associated with age-related diseases including Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) as well as cancers [46, 47][358–360]. Therefore, an effective mathematical model of the SUMOylation pathway in response to cellular stress can not only provide some new insights about SUMO-mediated regulatory mechanisms but also could lead to the development of new therapeutic strategies for preventing and/or treating those disease associated with cellular stress.

Chapter 5

Dynamic Modelling of SUMOylation in Cellular Response to Hypoxia

5.1 Introduction

Oxygen is a necessary component of aerobic species' life. Oxygen plays a crucial function since it is the ultimate acceptor of electrons in the mitochondrial respiratory chain. This enables for the last step of oxidative phosphorylation and the production of cellular energy ATP. The majority of processes required to sustain cellular viability consume ATP. Under order to live in normoxia, a cell must maintain a high and consistent ratio of cellular ATP/ADP. Cells that are reliant on a high continuous ATP/ADP ratio are reliant on oxygen. As a result, a reduction in normal oxygen supply (hypoxia) has an impact on cell survival [156][382]. Hypoxia is generally described as a decline in oxygen supply below the physiological level required to keep cellular functions. Hypoxia responses can also be classified according to temporal scales, including acute, intermediate, and chronic, as well as oxygen concentration levels, such as a moderate level (5-8% O_2), an anoxic level ($<$ 1% O_2) and normoxia condition (21% O_2) [383, 384]. Hypoxia-inducible factor 1 (HIF1), an oxygen-sensitive transcriptional activator, is the main mediator of this reaction. HIF1 is a transcription factor that facilitates the adaption and survival of cells and the entire organism from normoxia to hypoxia [385, 386]. HIF1α is considered to be the significant regulator in the HIF1 family. Post-translational modifications such as hydroxylation, ubiquitination, acetylation, and phosphorylation influence the stability and function of the

HIF1α. The HIF1α stays stable under hypoxia and interacts with coactivators response element-binding protein to control target gene expression. SUMOylation is one of the regulatory mechanisms proposed to adapt the activity of HIF1α to the needs of individual cells. SUMOylation has recognized as a crucial alteration in the physiological response to various stress, including hypoxia. SUMOylation processes are extremely dynamic, allowing for quick responses to environmental stimuli. The role of SUMOylation in the response to stress is a rapid growing subject of research. Regulation of a specific target protein's SUMOylation or the SUMO conjugation process adjustment lightly to change levels of global SUMOylation are two examples of adaptive response mechanisms [28][214]. The showing of enhanced global protein SUMOylation in vitro under hypoxia conditions provided the first indication that SUMOylation of proteins may be connected with changed cellular metabolic states in 2003 [317]. It has been suggested that elevated levels of protein SUMOylation in their brain and heart when mice were treated to whole animal hypoxia [38]. Furthermore, the discovery that hypoxia has a significant influence on cell biology and mammalian physiology via gene expression has generated a surge of interest in the biology of the HIF1 pathway and its relevance in human diseases like cancer. Emerging evidences indicated that low oxygen tension, or hypoxia, is one of the most notable and well-studied aspects of the tumour microenvironment. Therefore, understanding the hypoxia response and SUMOylation mechanisms is vital significant, which could provide a new perspective for developing potential therapeutic strategies for preventing or treating diseases associated with cellular stress, especially hypoxia. In this chapter, a combined mathematical modelling and experimental approach to investigate the role of HIF1α SUMOylation in response to hypoxia has been presented.

Understanding the complicated modulation of HIFs in both physiological and pathological processes has made significant progress [23][25, 26][161] in its mathematical modelling since seminal work has been published in [19]. By computing the integral of the ordinary differential equations (ODEs) for the factors associated with hypoxia, Kohn et al. [161] have predicted features of HRE occupancy of the switch-like response. Therefore, the computational model [25] is to propose a thorough quantification that describes the model's observed behaviour. This is accomplished by combining elementary pathway identification via extreme pathway analysis (EPA). AS a consequence of oxygen concentration, they investigated flux redistribution among the primary routes. In the case of hypoxia, the

findings show that the HIF1α process of excessive synthesis and degradation is the driving force behind the rapid reaction. In [26], the given computational model provided a new perspective on the molecular specifics of how cells perceive oxygen. It reveals how PHD2 can decide whether an acute hypoxia exposure results in a rapid or chronic regulation in hydroxylated HIF1α levels. Due to many studies have shown that it is impressive to take a consideration the SUMOylation mechanism into the response to hypoxia stress, particular SUMOylation of HIF1α. A dynamic model is proposed to simulate the role of HIF1α SUMOylation in response to hypoxia. In addition, an experiment is conducted for the purpose of validation of the presented model in terms of the SUMOylation levels under the hypoxia. SUMOylation of HIF1α, hydroxylation of HIF1α, SENP1 protein for activity and stability of HIF1α. To the best of the knowledge, this is the first attempt to develop the hypoxia response involved in SUMOylation of HIF1α by combining experiment and mathematical modelling. The biological process of SUMOylation and hydroxylation of HIF1α in response to hypoxia stress (block 3, block 4 and 5 in Figure 5.1) is proposed by the author, which is the innovation of the model. Subsequently, genetic algorithm (GA) and global sensitivity analysis are applied to the model for parameter optimisation and estimation.

The Chapter organisation is shown below. The introduction of the mathematical model of SUMOylation in response to hypoxia is presented in section 5.1. In section 5.2, the biological mechanisms and the novel mathematical modelling are described. In section 5.3, a genetic algorithm (GA) is applied to optimise the model parameters, and global sensitivity analysis is used to present the impact of input and output variables. In section 5.4, our experiments to validate the model are also displayed and employ the model to stimulate interesting scenarios that have not yet been tested experimentally. Finally discussions and conclusions are demonstrated in section 5.5.

5.2 Model Description

5.2.1 Model Overview—Biological Mechanism of Hypoxia

The dynamic model incorporates key molecular interactions in the HIFα pathway and the molecular components and steps of the model are shown in Figure 5.1. The biological

basis of the hypoxia response for our modelling, can be described as follows: in normoxia, HIF1α is hydroxylated by PHD, essential for the binding of the VHL tumor suppressor protein, and the degradation of ubiquitinated proteasome is then mediated by the VHL complex. As PHDs use oxygen as a cofactor for their hydroxylation activity, therefore, in hypoxia, the activity of PHD is inhibited and HIF1α is stabilized and accumulated (in Block 1 and 2). Thereby, HIFα is translocated to nucleus where these HIF1α concentrate on the nuclear stress granules and undergo multiple site SUMOylation (in Block 3) and deSUMOylation (in Block 4), which correlates with its stabilisation and transcriptional activities. Note that the SUMO E3 ligase PIASy is to enhance hypoxia-induced HIF1α SUMOylation and to negatively regulate its stability and transactivation. Furthermore, sentrin/SUMO specific protease 1 (SENP1), a deSUMOylation enzyme, stabilizes HIF1α and enhances its transcriptional activity by removing SUMO protein. And then heterodimerize with HIF1β (in Block 5). As s transcriptional factor, the heterodimer HIF (HIF1$\alpha \cdot$ HIF1β) recognizes and binds to the consensus sequence named hypoxia-responsive element (HRE) to activate the transcriptional activity of target genes (in Block 6).

The new hypoxia model is based on the underlying SUMOylation signalling network inferred from experimental findings, The dynamic model incorporates key molecular interactions in the HIF1α pathway and the molecular components and steps of the model are displayed in Figure 5.1. The biological basis of the hypoxia response for the modelling, can be described as follows: under normoxia conditions, HIF1α is hydroxylated by PHD, essential for the binding of the von Hippel-Lindau (VHL) tumor suppressor protein, and the degradation of ubiquitinated proteasome is then mediated by the VHL complex; As PHDs use oxygen as a cofactor for their hydroxylation activity, therefore, under hypoxia condition, the activity of PHD is inhibited and HIF1α is stabilized and accumulated in Block 1 and Block 2. Thereby, HIF1α is translocated to nucleus, the HIF1α concentrates on the nuclear stress granules and undergo multiple site SUMOylation (Block 3) and deSUMOylation (Block 4), which correlates with its stabilisation and transcriptional activities. Note that the SUMO E3 ligase is to enhance hypoxia-induced HIF1α SUMOylation and to negatively regulate its stability and transactivation. Furthermore, sentrin/SUMO Specific Protease 1 (SENP1), a deSUMOylation enzyme, stabilizes HIF1α and enhances its transcriptional activity by removing SUMO. Subsequently, HIF1β, one of subunits of hypoxia-inducible factors, binds to HIF1α to forms HIF1$\alpha \cdot$HIF1β complex in Block 5.

As a transcriptional factor, the heterodimer HIF1 (HIF1α·HIF1β) recognizes and binds to the consensus sequence named hypoxia-responsive element (HRE) to activate the transcriptional activity of target genes in Block 6.

To provide a quantitative framework for understanding the hypoxia pathway, the dynamic and ordinary differential equations-based model from the validated and published core components of the hypoxia response network is developed in Figure 5.1. The model integrates the current understanding of the interaction between the known HIF1α pathway components. In what follows, the detailed mathematical model for each of the blocks will be derived, where the mass action kinetics are assumed for the majority of the rate laws and the Michaelis-Menten kinetics are used be described the rate of enzymatic reactions involved.

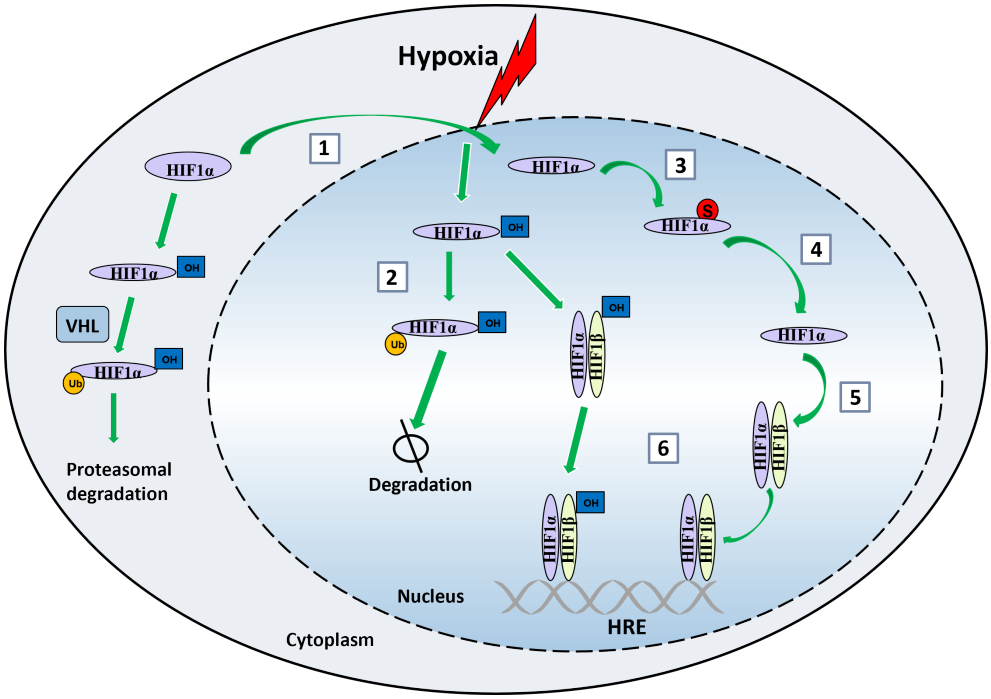

Figure 5.1. Biological mechanism of the cell response to hypoxia stress – OH: hydroxylation, ub: ubiquitination and s: SUMOylation

One of the most well-known models of enzyme kinetics in biology is the Michaelis-Menten kinetics. A mathematical model of the reaction is proposed by [362] in 1913. It includes an enzyme E, forming a complex ES, with a substrate S and releasing a product P, which regenerates the original enzyme. This can be diagrammatically expressed as

$$[\mathrm{E}] + [\mathrm{S}] \underset{\mathrm{k}_r}{\overset{\mathrm{k}_f}{\rightleftharpoons}} [\mathrm{E}\cdot\mathrm{S}] \xrightarrow{\mathrm{k_{cat}}} [\mathrm{E}] + [\mathrm{P}] \tag{5.1}$$

where k_f denotes forward rate constant, k_r means reverse rate constant and k_{cat} shows the catalytic rate constant.

A set of four nonlinear ordinary differential equations that specify the rate of change of reactants with time t are produced by using the law of mass action, which provides that the rate of a reaction is proportional to the product of the concentrations of the reactants (i.e. [E][S]) [363].

The reaction can be described with four differential equations

$$\frac{\mathrm{d}[E]}{\mathrm{d}t} = -k_f[E][S] + k_r[E \cdot S] + k_{cat}[E \cdot S] \tag{5.2}$$

$$\frac{\mathrm{d}[S]}{\mathrm{d}t} = -k_f[E][S] + k_r[E \cdot S] \tag{5.3}$$

$$\frac{\mathrm{d}[E \cdot S]}{\mathrm{d}t} = -k_f[E][S] - k_r[E \cdot S] - k_{cat}[E \cdot S] \tag{5.4}$$

$$\frac{\mathrm{d}[P]}{\mathrm{d}t} = k_{cat}[E \cdot S] \tag{5.5}$$

In reaction (5.6), it shows the HIF1α degradation, which are eliminated in some cases, because they are supposed to escape the process shortly after production and have no effect on the dynamics

$$[\mathrm{HIF1}\alpha] \xrightarrow{\mathrm{k}_{1d}} [\phi] \tag{5.6}$$

Under normoxia conditions, HIF1α prolyl hydroxylation by PHD, which forms the complex (HIF1α·PHD), very substantially increases its binding to the VHL. When PHD catalysis is limited by oxygen availability, i.e. in hypoxia, the degradation of HIF1α is slowed, its stabilisation and level rises, it dimerizes with HIF1β and upregulates HIF-target gene transcription.

$$[\mathrm{HIF1}\alpha] + [\mathrm{PHD}] \underset{\mathrm{k}_{2r}}{\overset{\mathrm{k}_{2f}}{\rightleftharpoons}} [\mathrm{HIF1}\alpha \cdot \mathrm{PHD}] \tag{5.7}$$

After then, with participation of the oxygen, HIF1α is hydroxylated to form HIF1α_{OH}

$$[\mathrm{HIF1}\alpha \cdot \mathrm{PHD}] + [\mathrm{O_2}] \xrightarrow{\mathrm{k}_3} [\mathrm{HIF1}\alpha_{\mathrm{OH}}] + [\mathrm{PHD}] \tag{5.8}$$

The hydroxylated HIF1α (HIF1α_{OH}) binds to VHL, with ability to enrich E3 ubiquitin ligase complex and thus mediates rapid degradation of HIF1α by the proteasome. After

this pathway, VHL are released. Reaction is derived as below

$$[\text{HIF1}\alpha_{\text{OH}}] + [\text{VHL}] \underset{\text{k}_{4r}}{\overset{\text{k}_{4f}}{\rightleftharpoons}} [\text{HIF1}\alpha_{\text{OH}} \cdot \text{VHL}] \xrightarrow{\text{k}_{4cat}} [\text{VHL}] \tag{5.9}$$

Hypoxia promotes translocation of HIF1α to the nucleus to facilitate its binding to PIASy (Protein inhibitor of activated STAT) as a specific E3 ligase for hypoxia-induced HIF1α SUMOylation, enabling the conjugation of HIF1α by SUMO. Interaction of PIASy and HIF1α is essential for hypoxia-induced HIF1α SUMOylation. The SUMOylation pathway consists three specific chemical reactions, which is associated with several specific enzymes (*e.g. E1, E2* and *SENP*), and will be discussed in some details below.

The reaction (5.10) describes that the inactive sumo precursor (presumo) is cleaved by specific enzyme SENP, and converts the presumo into a mature form, the presumo·SENP is protein synthesis in the pathway

$$[\text{presumo}] + [\text{SENP}] \underset{\text{k}_{5r}}{\overset{\text{k}_{5f}}{\rightleftharpoons}} [\text{presumo} \cdot \text{SENP}] \xrightarrow{\text{k}_{5cat}} [\text{sumo}] + [\text{SENP}] \tag{5.10}$$

$$[\text{sumo}] + [\text{E1}] \xrightarrow{\text{k}_6} [\text{sumo} \cdot \text{E1}] \tag{5.11}$$

$$[\text{sumo} \cdot \text{E1}] + [\text{E2}] \xrightarrow{\text{k}_7} [\text{sumo} \cdot \text{E2}] + [\text{E1}] \tag{5.12}$$

The reaction (5.13) and (5.14) indicate the ligation steo in SUMOylation. E3, supported by E2, attached the ubiquitin to a lysine residue of the substrate protein or a lysine residue of the attached ubiquitin. E3, plays a key role in the recognition of the amino acid sequence of a target protein (HIF1α). sumo·E2 binds to unmodified target protein HIF1α, and forms SUMOylated target protein HIF1α_s, then, E2 and E3 are released at the same time

$$[\text{HIF1}\alpha] + [\text{E3}] \underset{\text{k}_{8r}}{\overset{\text{k}_{8f}}{\rightleftharpoons}} [\text{HIF1}\alpha_s \cdot \text{E3}] \tag{5.13}$$

$$[\text{HIF1}\alpha \cdot \text{E3}] + [\text{sumo} \cdot \text{E2}] \xrightarrow{\text{k}_9} [\text{E2}] + [\text{E3}] + [\text{HIF1}\alpha_s] \tag{5.14}$$

The reaction (5.15) proposes the deSUMOylation process

$$[\mathrm{HIF1}\alpha_s] + [\mathrm{SENP}] \underset{\mathrm{k}_{10r}}{\overset{\mathrm{k}_{10f}}{\rightleftharpoons}} [\mathrm{HIF1}\alpha_s \cdot \mathrm{SENP}] \xrightarrow{\mathrm{k}_{10cat}} [\mathrm{HIF1}\alpha] + [\mathrm{sumo}] + [\mathrm{SENP}] \quad (5.15)$$

One part of the HIF1β is bound to un-hydroxylated HIF1α to produce the HIF1α·HIF1β in reaction (5.16), the other part is combined with hydroxylated HIF1α (HIF1α_{OH}) forms the synthesis (HIF1α_{OH}·HIF1β) in reaction (5.17)–(5.19)

$$[\mathrm{HIF1}\alpha] + [\mathrm{HIF1}\beta] \underset{\mathrm{k}_{11r}}{\overset{\mathrm{k}_{11f}}{\rightleftharpoons}} [\mathrm{HIF1}\alpha \cdot \mathrm{HIF1}\beta] \quad (5.16)$$

$$[\mathrm{HIF1}\alpha] + [\mathrm{HIF1}\beta] + [\mathrm{PHD}] \underset{\mathrm{k}_{12r}}{\overset{\mathrm{k}_{12f}}{\rightleftharpoons}} [\mathrm{HIF1}\alpha \cdot \mathrm{HIF1}\beta \cdot \mathrm{PHD}] \quad (5.17)$$

$$[\mathrm{HIF1}\alpha \cdot \mathrm{HIF1}\beta \cdot \mathrm{PHD}] + [\mathrm{O_2}] \xrightarrow{\mathrm{k}_{13}} [\mathrm{HIF1}\alpha_{\mathrm{OH}} \cdot \mathrm{HIF1}\beta] \quad (5.18)$$

$$[\mathrm{HIF1}\alpha_{\mathrm{OH}}] + [\mathrm{HIF1}\beta] \underset{\mathrm{k}_{14r}}{\overset{\mathrm{k}_{14f}}{\rightleftharpoons}} [\mathrm{HIF1}\alpha_{\mathrm{OH}} \cdot \mathrm{HIF1}\beta] \quad (5.19)$$

As mentioned above, the block 6 shows the heterodimer HIF1 (HIF1α·HIF1β/HIF1α_{OH}·HIF1β) recognizes and binds to the consensus sequence named hypoxia-responsive element (HRE) to activate the transcriptional activity of target genes. The HRE acts as a promoter for hypoxia-regulated genes and its occupancy modulates the levels of these genes expression. The reactions are given as follows

$$[\mathrm{HIF1}\alpha \cdot \mathrm{HIF1}\beta] + [\mathrm{HRE}] \underset{\mathrm{k}_{15r}}{\overset{\mathrm{k}_{15f}}{\rightleftharpoons}} [\mathrm{HIF1}\alpha \cdot \mathrm{HIF1}\beta \cdot \mathrm{HRE}] \quad (5.20)$$

$$[\mathrm{HIF1}\alpha_{\mathrm{OH}} \cdot \mathrm{HIF1}\beta] + [\mathrm{HRE}] \underset{\mathrm{k}_{16r}}{\overset{\mathrm{k}_{16f}}{\rightleftharpoons}} [\mathrm{HIF1}\alpha_{\mathrm{OH}} \cdot \mathrm{HIF1}\beta \cdot \mathrm{HRE}] \quad (5.21)$$

5.2.2 The Mathematical Modelling of Hypoxia

In hypoxia, PHD is inactivated, leading to HIF1α protein stabilisation and translocation to the nucleus. PHD is assumed to translocate in and out of the nucleus. In Block 1, it shows HIF1α is hydroxylated by PHDs in an oxygen-dependent manner. From Eqs. (5.22),

which as a prolylhyroxylase, can be given by

$$\begin{aligned}\frac{\mathrm{d}[HIF1\alpha]}{\mathrm{d}t} =& -k_{1d}[HIF1\alpha] - k_{2f}[HIF1\alpha][PHD] \\ &+ k_{2r}[HIF1\alpha \cdot PHD] - k_{11f}[HIF1\alpha][HIF1\beta] \\ &+ k_{11r}[HIF1\alpha \cdot HIF\beta]\end{aligned} \tag{5.22}$$

$$\begin{aligned}\frac{\mathrm{d}[PHD]}{\mathrm{d}t} =& -k_{2f}[HIF1\alpha][PHD] + (k_{2r} + k_3[O_2])[HIF1\alpha \cdot PHD] \\ &- k_{12f}[HIF1\alpha \cdot HIF1\beta][PHD] \\ &+ (k_{12r} + k_{17}[O_2])[HIF1\alpha \cdot HIF\beta \cdot PHD]\end{aligned} \tag{5.23}$$

The HIF1α·PHD as intermediate product, which exists in the synthetic process. The complex of HIF1α and PHD is calculated with a mass balance

$$\frac{\mathrm{d}[HIF1\alpha \cdot PHD]}{\mathrm{d}t} = k_{2f}[HIF1\alpha][PHD] - (k_{2r} + k_3[O_2])[HIF1\alpha \cdot PHD] \tag{5.24}$$

With the participation of oxygen, the HIF1α_{OH} is produced after prolyl hydroxylation. It could also bind to VHL and HIF1β in the subsequent reactions

$$\begin{aligned}\frac{\mathrm{d}[HIF1\alpha_{OH}]}{\mathrm{d}t} =& k_3[HIF1\alpha][O_2][HIF1\alpha \cdot PHD] - k_{4f}[HIF1\alpha_{OH}][VHL] \\ &+ k_{4r}[HIF1\alpha_{OH} \cdot VHL] - k_{14f}[HIF1\alpha_{OH}][HIF1\beta] \\ &+ k_{14r}[HIF1\alpha_{OH} \cdot HIF1\beta]\end{aligned} \tag{5.25}$$

In the model, the VHL level can be obtained with

$$\frac{\mathrm{d}[VHL]}{\mathrm{d}t} = -k_{4f}[HIF1\alpha_{OH}][VHL] + (k_{4r} + k_{4cat})[HIF1\alpha_{OH} \cdot VHL] \tag{5.26}$$

The level of hydroxylated HIF1α and VHL complex (HIF1α_{OH}·VHL is given

$$\frac{\mathrm{d}[HIF1\alpha_{OH} \cdot VHL]}{\mathrm{d}t} = k_{4f}[HIF1\alpha_{OH}][VHL] - (k_{4r} + k_{4cat})[HIF1\alpha_{OH} \cdot VHL] \tag{5.27}$$

5.2.3 The Mathematical Model of SUMOylation of the hydroxylated HIF1α

In the new hypoxia model, the hydroxylated HIF1α is the target protein and modified by sumo protein. The effect of enzyme E3 is crucial for the hypoxia response model. Parameters for forward reaction and reverse reaction (k_f, k_r) can be derived by Michaelis-Menten constant k_M and k_{cat}.

The presumo is inactive form of sumo, it need to change in mature sumo form to obtain the level of presumo, which interacts with enzyme SENP.

$$\frac{d[presumo]}{dt} = -k_{5f}[presumo][SENP] + k_{5r}[presumo \cdot SENP] \tag{5.28}$$

The enzyme SENP is not only plays a role in converting presumo into sumo process, but also related to deSUMOylation part. In deSUMOylation, it dissociates the complex of HIF1α_s·SENP and free HIF1α and free sumo are obtained finally

$$\begin{aligned}\frac{d[SENP]}{dt} =& k_{5f}[presumo][SENP] + k_{5r}[presumo \cdot SENP] \\ &+ k_{5cat}[presumo \cdot SENP] - k_{10f}[SENP][HIF1\alpha_s] \\ &+ k_{10r}[SENP \cdot HIF1\alpha_s] + k_{10cat}[SENP \cdot HIF1\alpha_s]\end{aligned} \tag{5.29}$$

The presumo·SENP is an intermediate product, it exists briefly and then immediately changes in another form

$$\begin{aligned}\frac{d[presumo \cdot SENP]}{dt} =& k_{5f}[presumo][SENP] - k_{5r}[presumo \cdot SENP] \\ &- k_{5cat}[presumo \cdot SENP]\end{aligned} \tag{5.30}$$

In SUMOylation process, sumo protein will be degraded, and the rest level of them is associated with next reaction, including activation and ligation process

Where F(T) is the temperature and $[*]$ denotes the concentration of $*$, as a function of time(t)

$$\begin{aligned}\frac{d[sumo]}{dt} =& k_{5cat}[presumo \cdot SENP] - k_6[sumo][E1] \\ &+ k_{10cat}[SENP \cdot HIF1\alpha_s] + F(T)\end{aligned} \tag{5.31}$$

The level of E1, which as a activation enzyme, activating sum in an ATP-dependent manner

$$\frac{\mathrm{d}[E1]}{\mathrm{d}t} = -k_6[sumo][E1] + k_7[sumo \cdot E1][E2] \tag{5.32}$$

For the intermediate product, sumo·E1 is calculated with a mass balance

$$\frac{\mathrm{d}[sumo \cdot E1]}{\mathrm{d}t} = k_6[sumo][E1] - k_7[sumo \cdot E1][E2] \tag{5.33}$$

E2 as a conjugating enzyme in the sumo conjugation step

$$\frac{\mathrm{d}[E2]}{\mathrm{d}t} = -k_7[sumo \cdot E1][E2] + k_9[sumo \cdot E2][HIF1\alpha \cdot E3] \tag{5.34}$$

and

$$\frac{\mathrm{d}[sumo \cdot E2]}{\mathrm{d}t} = k_7[sumo \cdot E1][E2] - k_9[sumo \cdot E2][HIF1\alpha \cdot E3] \tag{5.35}$$

The ligation of sumo and target HIF1α occurs with E3 as a ligating enzyme

$$\begin{aligned}\frac{\mathrm{d}[HIF1\alpha \cdot E3]}{\mathrm{d}t} =& k_{8f}[HIF1\alpha][E3] - k_{8r}[HIF1\alpha \cdot E3] \\ & - k_9[sumo \cdot E2][HIF1\alpha \cdot E3]\end{aligned} \tag{5.36}$$

HIF1α is transferred for the SUMOylation. The sumo protein is released by the synthesis sumo·E2, and then the sumo binds to target HIF1α to form HIF1α_s. Besides, the degradation of HIF1α_s happens at the same time with the formation of HIF1α_s

$$\begin{aligned}\frac{\mathrm{d}[HIF1\alpha_s]}{\mathrm{d}t} =& k_9[sumo \cdot E2][HIF1\alpha \cdot E3] - k_{10f}[HIF1\alpha_s][SENP] \\ & + k_{10r}[HIF1\alpha_s \cdot SENP]\end{aligned} \tag{5.37}$$

HIF1α_s·SENP is the intermediate product of the deSUMOylation process, SENP is related in the step as a specific enzyme to remove sumo proteins from the HIF1α_s, and form the HIF1α

$$\begin{aligned}\frac{\mathrm{d}[HIF1\alpha_s \cdot SENP]}{\mathrm{d}t} =& k_{10f}[HIF1\alpha_s][SENP] + k_{10r}[HIF1\alpha_s \cdot SENP] \\ & - k_{10cat}[HIF1\alpha_s \cdot SENP]\end{aligned} \tag{5.38}$$

The expression of HIF1β, HIF1α·HIF1β, HIF1α·HIF1β·PHD and HIF1α_{OH}·HIF1β are displayed below

$$\begin{aligned}\frac{\mathrm{d}[HIF1\beta]}{\mathrm{d}t} =& -k_{11f}[HIF1\alpha][HIF1\beta]+k_{11r}[HIF1\alpha\cdot HIF1\beta]\\ &-k_{14f}[HIF1\alpha_{OH}][HIF1\beta]+k_{11r}[HIF1\alpha_{OH}\cdot HIF1\beta]\end{aligned} \tag{5.39}$$

$$\begin{aligned}\frac{\mathrm{d}[HIF1\alpha\cdot HIF1\beta]}{\mathrm{d}t} =& k_{11f}[HIF1\alpha][HIF1\beta]-k_{11r}[HIF1\alpha\cdot HIF1\beta]\\ &-k_{12f}[HIF1\alpha\cdot HIF1\beta][PHD]+k_{12r}[HIF1\alpha\cdot HIF1\beta\cdot PHD]\\ &-k_{15f}[HIF1\alpha\cdot HIF1\beta][HRE]+k_{15r}[HIF1\alpha\cdot HIF1\beta\cdot HRE]\end{aligned} \tag{5.40}$$

$$\begin{aligned}\frac{\mathrm{d}[HIF1\alpha\cdot HIF1\beta\cdot PHD]}{\mathrm{d}t} =& -k_{12f}[HIF1\alpha\cdot HIF1\beta][PHD]\\ &+(k_{12r}+K_{13}[O_2])[HIF1\alpha\cdot HIF1\beta\cdot PHD]\end{aligned} \tag{5.41}$$

$$\begin{aligned}\frac{\mathrm{d}[HIF1\alpha_{OH}\cdot HIF1\beta]}{\mathrm{d}t} =& k_3[O_2][HIF1\alpha\cdot HIF1\beta]+k_{14f}[HIF1\alpha_{OH}][HIF1\beta]\\ &-k_{14r}[HIF1\alpha_{OH}\cdot HIF1\beta]-k_{16f}[HIF1\alpha_{OH}\cdot HIF1\beta][HRE]\\ &+k_{16r}[HIF1\alpha_{OH}\cdot HIF1\beta\cdot HRE]\end{aligned} \tag{5.42}$$

The different heterodimers HIF1 (HIF1α_s·HIF1β/HIF1$\alpha_O H$·HIF1β) occupied HRE for genes expression

$$\begin{aligned}\frac{\mathrm{d}[HRE]}{\mathrm{d}t} =& -k_{15f}[HIF1\alpha\cdot HIF1\beta][HRE]+k_{15r}[HIF1\alpha\cdot HIF1\beta\cdot HRE]\\ &-k_{16f}[HIF1\alpha_{OH}\cdot HIF1\beta][HRE]+k_{16r}[HIF1\alpha_{OH}\cdot HIF1\beta\cdot HRE]\end{aligned} \tag{5.43}$$

The occupation of HRE by HIF1α·HIF1β, the complexes of HIF1α·HIF1β and HRE is calculated with the following rate laws

$$\begin{aligned}\frac{\mathrm{d}[HIF1\alpha \cdot HIF1\beta \cdot HRE]}{\mathrm{d}t} =& k_{15f}[HIF1\alpha \cdot HIF1\beta][HRE] \\ & - k_{15r}[HIF1\alpha \cdot HIF1\beta \cdot HRE]\end{aligned} \tag{5.44}$$

The occupation of HRE by HIF1α_{OH}·HIF1β is showed below

$$\begin{aligned}\frac{\mathrm{d}[HIF1\alpha_{OH} \cdot HIF1\beta \cdot HRE]}{\mathrm{d}t} =& k_{16f}[HIF1\alpha_{OH} \cdot HIF1\beta][HRE] \\ & - k_{16r}[HIF1\alpha_{OH} \cdot HIF1\beta \cdot HRE]\end{aligned} \tag{5.45}$$

5.3 Methodology

5.3.1 Model Parameter Optimization

A general challenge in modelling biological systems is the identification of system parameters because often these are not directly accessible experimentally [16][364–367]. In the study, the parameters (a total of 29) include forward and reverse reaction rates corresponding to the reactions, the temperature constants, and translation rate etc. in the Eqs. (5.22)–(5.25). Eqs. (5.26)–(5.45) derived in the previous section. None of these rate constants are explicitly known but still for some of them a reasonable range can be estimated from databases [368]. The author first manually tuned the parameters to produce the qualitative behaviour of the system with the baselines and the reasonable ranges of the parameters taken from literature [19][23][25, 26]. These parameters then were used as a starting point for a deeper investigation of the parameter space represented by the 29 rate constants. More specifically, in this study, we estimated the model parameters by using a genetic algorithm (GA) based data-driven approach. Differential evolution is a population-based heuristic search algorithm that optimises a problem by iteratively improving a candidate solution based on an evolutionary process [369, 370]. Such algorithms make few or no assumptions about the underlying optimization problem and can quickly explore very large design spaces. Because the GA is a data-driven algorithm, we defined an objective function reflecting the quality of the fit by a root mean square error (RMSE) of the deviations between model simulations and data taken from literature [9][36] and

our experiments. GA method was then applied to find a set of parameters which locally optimises the objective function. Since the training data is relative values so in this study all the data were normalized between 0 (0%) and 1 (100%). For the GA method, the GA parameters are defined, as follows: the population size was 10, the crossover probability was 0.94, the mutation probability was 0.08. The smallest RMSE in each population was selected and the iteration was terminated when the fitness converged. In order to validate the model, the author then carried out experiments to generate data to described in the Model verification section. The obtained data, which has been shown in Figure 5.2.

5.3.2 Model Verification by Experimental Data

HeLa cells were maintained in 12-well plates with Dubecco's Modified Eagle's Medium (DMED) containing 10% fetal bovine serum (FBS), 5mM glutamine, and 100U/mL penicillin-streptomycin abd incubated at 37 °C in humidified air with 5% CO_2 levels. When the cells reached confluence, the culture medium were replaced with fresh degassed culture medium and cultured in a hypoxic (1%O_2; 5%CO_2; 37 °C) incubator while their control cells were cultured in normoxic (ambient air; 5%CO_2; 37 °C) for indicated time duration.

Cell lysate samples were prepared using a Triton X-100 lysis buffer (TLB) on ice with three additional reagents in order to detect SUMOylation: protease inhibitor at 1:50 dilution (Roche tablet EDTA-free Protease Inhibitor Cocktail 1:1000 in water); 0.1% solidum dodecyl sulphate (SDS); and 20mM N-ethylmaleimide (NEM) added to the lysis buffer immediately before use.

Briefly, at the end of culture duration, the cells were removed from the incubator and spun down at 4,000 rpm for 2 mins. Excess media was removed from the Eppendorf tubes and 60μL TLB was piptted into each tube. The cells were kept for 15 mins on ice for lysis. The cell lysates were then sonicated and spun down for 15 mins at 4 °C and 13,200 rpm, and transferred into new Eppendorf tubes. 6×Laemmli buffer was added to each tube. The samples were stored at -80 °C until further use.

Western blotting was conducted to compare relative levels of HIF1α, SENP1 related to loading control β-Actin between experimental conditions.

Bond Breaker^{TM} TCEP (1:10; Sigma) was added to aliquots of each cell lysate sample.

The samples were spun down for 30 secs at 13,200 rpm, vortexed briefly, and heated at 70°C for 10 mins. SDS-PAGE (polyacrylamide gel electrophoresis) was performed to separate protein bands. Proteins were transferred onto Immobilon®-FL PVDF membranes (Millipore). Immunoblotting was performed on the membranes to detect SUMO2/3 levels using primary antibodies for HIF1α (610958; BD Transduction Laboratories™, mouse), SENP1 (ab108981; Abcam; Rabbit) and β-Actin (Proteintech; mouse).
Antibody complexes were detected with HRP-conjugated secondary antibodies (Sigma) or IRDye 800CW/68oRD near-infrared fluorescent secondary antibodies (LI-COR Bioscience, Ltd.) and then developed by chemiluminescence using Luminata™ ECL™ Prime or Select Western Blotting Detection Reagent (GE Healthcare Life Sciences) on film (CL-XPosure™ Film, Thermo Scientific™) or scanned with Li-COR Odyssey machine. ImageJ and Image Studio Lite were used for protein quantifications, respectively.

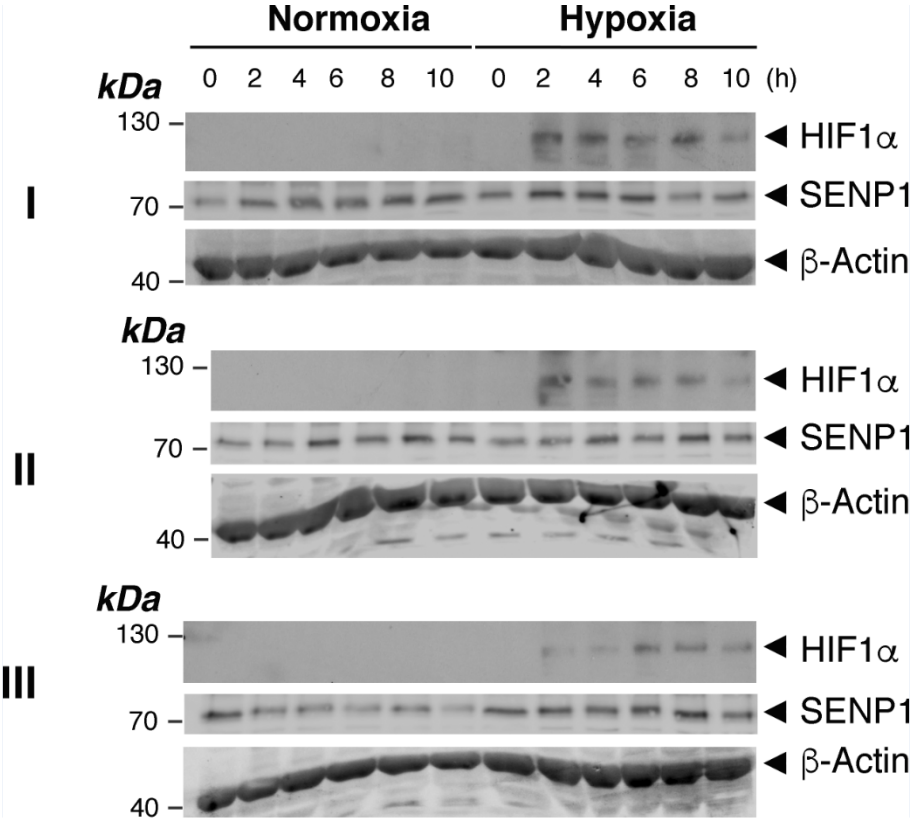

Figure 5.2. Western-blotting data from our hypoxia experiments

5.3.3 Global Sensitivity Analysis

In this chapter, a variance-based Sobol's method is used to investigate the influence of model parameters and their interactions on output variables in a mathematical model of hypoxia. Variance-based sensitivity analysis is a form of global sensitivity analysis (GSA). The results of Sobol's method for GSA are described in section 5.4.2.

5.4 Results

5.4.1 Simulation and Experiment Results

In order to test the hypoxia model, the primary synthesis level of dynamic model which triggered by low oxygen tension as presented in Figures 5.3-5.6. The total simulation time is 10 hours. The baseline values of the parameters used for the model is shown in Table 5.1.

Table 5.1. The baseline values of the parameters of the model

Parameters	Parameter Description	Values
k_{1d}	Rate of HIF1α degradation	0.0007 [19]
k_{2f}	Rate of HIF1α and PHD association	1.5478 [19]
k_{2r}	Rate of HIF1α·PHD complex dissociation	0.0416 [19]
k_3	Formation rate of HIF1α_{OH}	0.0226 [19]
k_{4f}	Rate of HIF1α_{OH} and VHL association	0.4738 [19]
k_{4r}	Rate of HIF1α_{OH}·VHL complex dissociation	0.1392 [19]
k_{4cat}	Catalytic rate of HIF1α_{OH}·VHL complex	0.2144 [19]
k_{5f}	Rate of HIF1α_{OH} and VHL association	0.4738
k_{5r}	Rate of HIF1α_{OH}·VHL complex dissociation	0.1392 [19]
k_{5cat}	Catalytic rate of HIF1α_{OH}·VHL complex	0.2144
k_6	Rate of sumo and E1 association	0.926
k_7	Rate of E1 dissociation and formation of sumo·E2 complex	0.101
k_{8f}	Rate of HIF1α and E3 association	0.001
k_{8r}	Rate of HIF1α·E3 complex dissociation	0.37
k_9	Rate of E2 dissociation from sumo, E3 dissociation from HIF1α and formation of sumo·HIF1α complex HIF1α_s	0.00034
k_{10f}	Rate of HIF1α_s and SENP association	10
k_{10r}	Rate of HIF1α_{OH}·VHL complex dissociation	33.1
k_{10cat}	Catalytic rate of HIF1α_{OH}·VHL complex	50.4
k_{11f}	Rate of HIF1α and HIF1β association	0.0148
k_{11r}	Rate of HIF1α·HIF1β complex dissociation	0.6163
k_{12f}	Rate of HIF1α and HIF1β and PHD association	1.5478 [19]
k_{12r}	Rate of HIF1α·HIF1β·PHD complex dissociation	0.0416 [19]
k_{13}	Rate of HIF1α·HIF1β·PHD and O_2 association	0.0226 [19]
k_{14f}	Rate of HIF1α_{OH} and HIF1β association	0.0148
k_{14r}	Rate of HIF1α_{OH}·HIF1β complex dissociation	1.6733
k_{15f}	Rate of HIF1α·HIF1β and HRE association	0.2681
k_{15r}	Rate of HIF1α·HIF1β·HRE complex dissociation	0.0809
k_{16f}	Rate of HIF1α_{OH}·HIF1β and HRE association	0.2681
k_{16r}	Rate of HIF1α_{OH}·HIF1β·HRE complex dissociation	0.0809

The Figure 5.3 shows that SUMO conjugation (HIF1α_s) level under 1% oxygen tension. The HIF1α_s level risen gradually in around 6 h, which demonstrates that SUMO proteins gradually conjugate to substrate. After that the level of SUMO conjugation remains stable, which indicates that hypoxia might increase the stability of HIF1α.

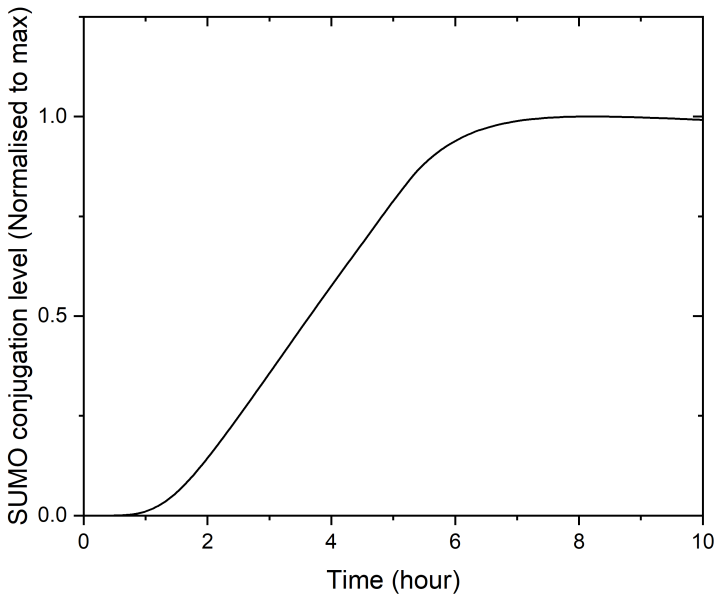

Figure 5.3. SUMOylated HIF1α (HIF1α_s) level under hypoxia stress

The Figure 5.4 demonstrates that SUMO conjugation (HIF1aα_s) level under three different oxygen tensions. It can be observed that the SUMO conjugation level increased individually from 0 to 6 h, after that the level of SUMO conjugation tendency stabilise and maintain at a steady state. The lower oxygen tension, the higher SUMO conjugation level. Under normal hypoxia condition (0.5%-10%), the HIF1α level is accumulated. Therefore, the SUMOylated HIF1α level is increased at the same time.

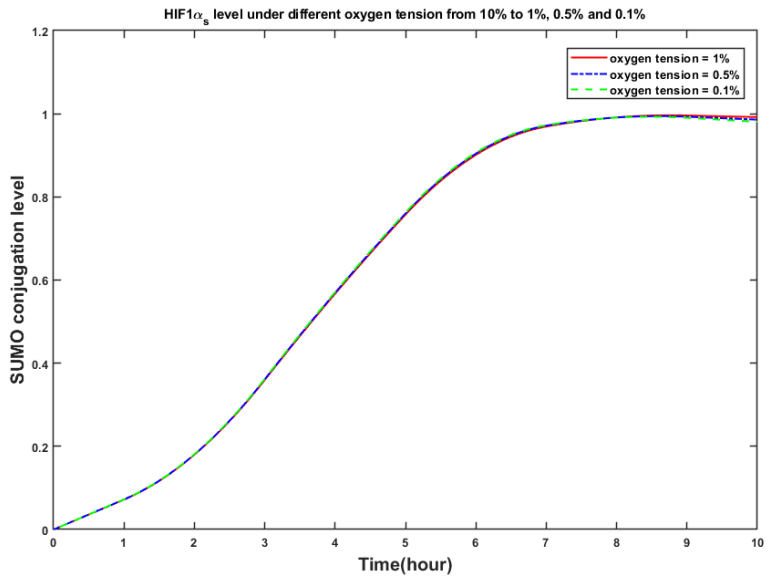

Figure 5.4. HIF1α_s level under different oxygen tension from 10% to 1%, 0.5% and 0.1%

The SENP1 level induced by 1% oxygen tension of hypoxia condition has shown in Figure 5.5. The SENP1 level is markedly increased in cells after hypoxia treatment at start and get maximum value around 6 h, and then reducing.

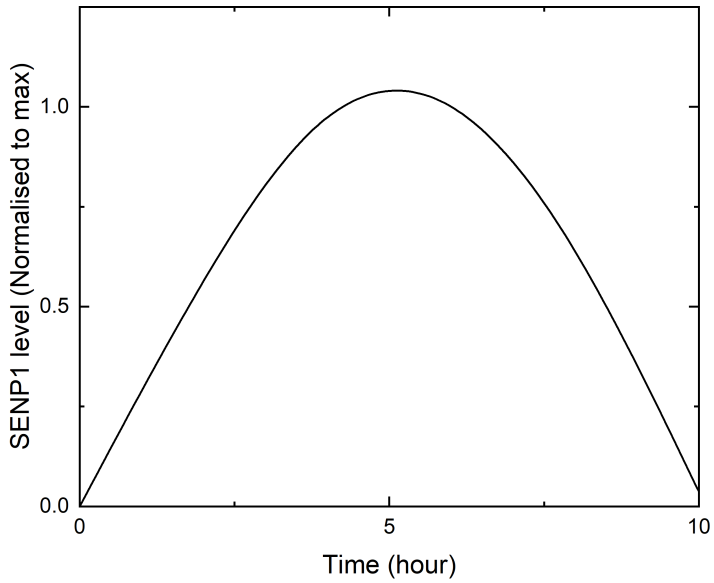

Figure 5.5. SENP1 level under hypoxia stress

The comparison of SENP1 level between simulation result and experimental data is presented in Figure 5.6. The root mean square error (RMSE) between the simulation level

and experimental result is 0.14. It can be given from the Figure 5.6, the concentration of SENP1 protein starts to increase and approach the peak in around 6 h, which implies that hypoxia could induce the SENP1 expression and would further enhance the HIF1α activity. The level of SENP1 reduces gradually afterwards.

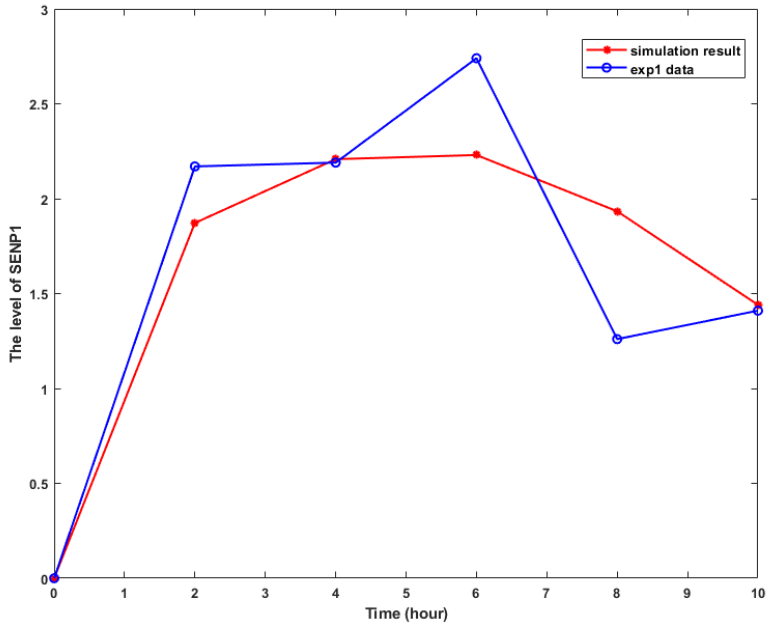

Figure 5.6. Comparison of SENP1 level under different experiments

5.4.2 GSA Computational Results for Hypoxia Model

In this section, the GSA (Sobol's method) results of hypoxia model are presented below. In GSA, the model output uncertainty is decomposed and then assigned to its input parameters. Using the algorithm of Sobol's method, two sensitivity indices has been obtained: first-order sensitivity index or main effect index (S_i) and total-order sensitivity index or interaction effect index (S_{Ti}). The results of both sensitivity indices are displayed in Figure 5.7-Figure 5.17 for N=5000 model runs per parameter.

Figure 5.7 shows that the parameter k_{2f}, k_{12f} and k_{16f} have both strong main effects and interaction effects on PHD among other parameters.

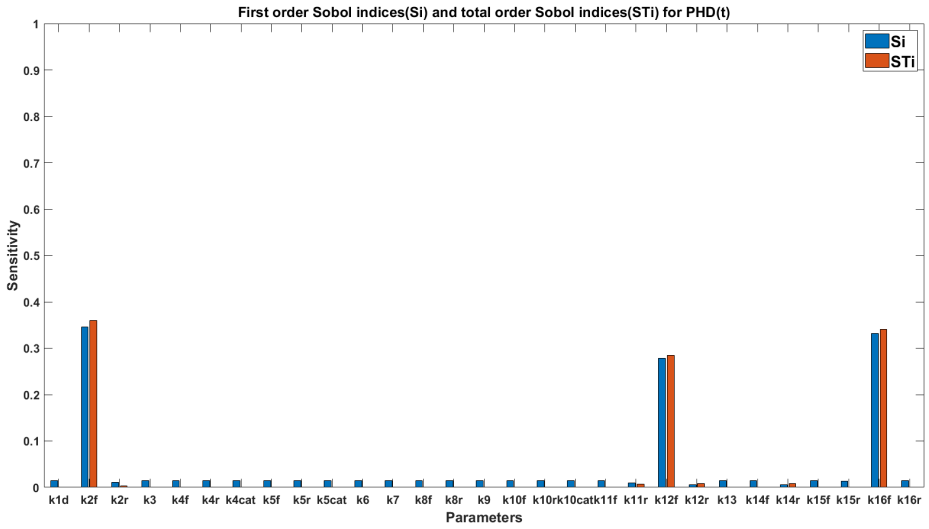

Figure 5.7. First- and Total-order of Sobol's Indices for PHD

The parameters k_{4f}, k_{14r}, k_{15r} and k_{16f} indicated major contributions of main and interaction effects in output for VHL, while k_{2f} and k_{12f} have smaller effects on VHL as shown in Figure 5.8.

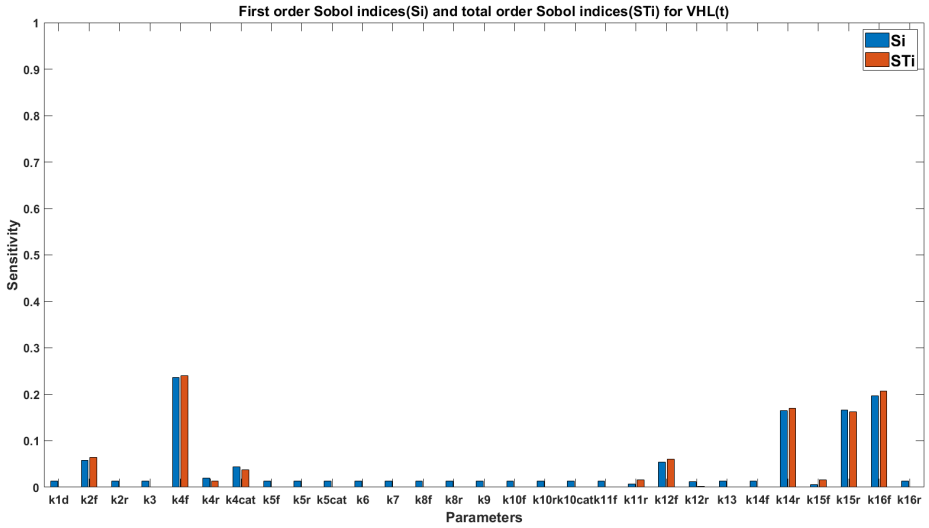

Figure 5.8. First- and Total-order of Sobol's Indices for VHL

Figure 5.9 demonstrates that k_{2f}, k_{11f}, k_{12f} and k_{16f} are most influential on both main and interaction effects than on other parameters for the HIF1α.

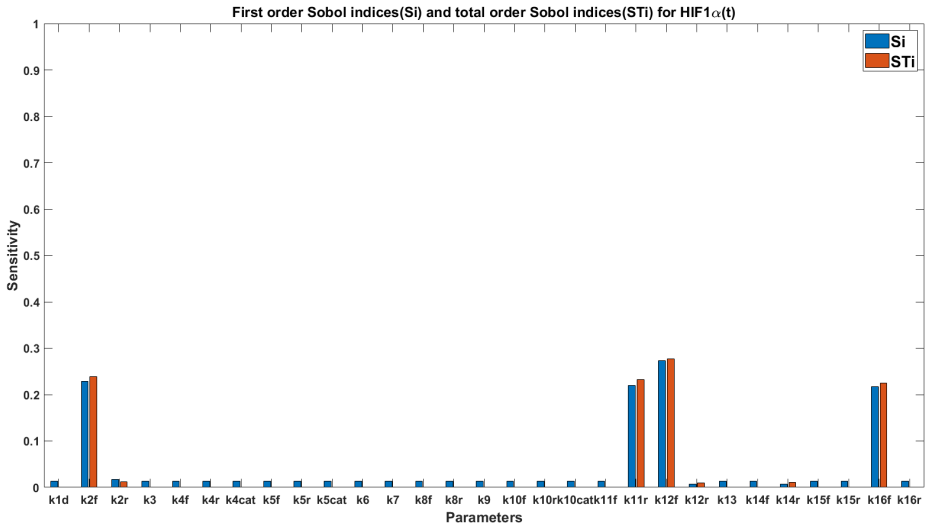

Figure 5.9. First- and Total-order of Sobol's Indices for HIF1α

Figure 5.10 demonstrates that k_{2f}, k_{12f}, k_{14f} and k_{16f} are most influential on both main and interaction effects than on other parameters for the HIF1α_{OH}.

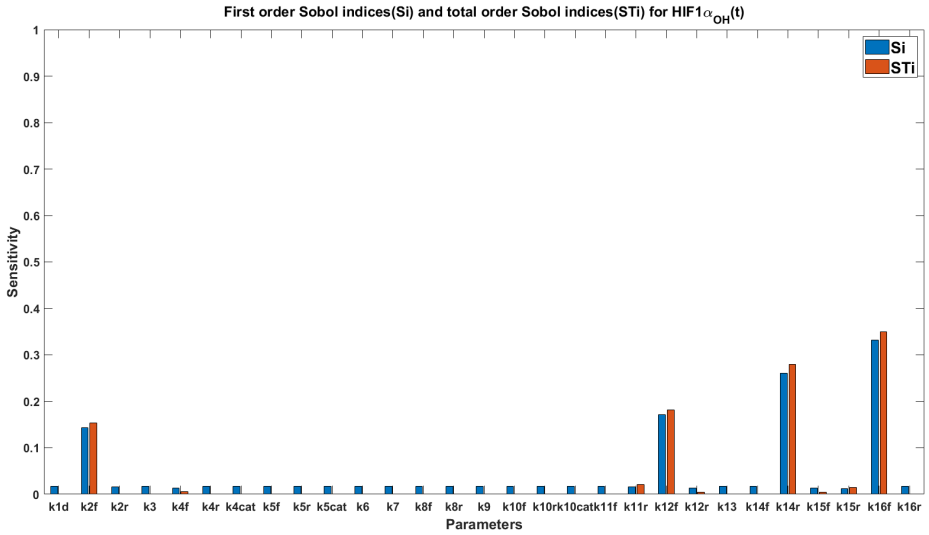

Figure 5.10. First- and Total-order of Sobol's Indices for HIF1α_{OH}

The parameters k_{2f}, k_{12f} and k_{16f} indicated major contributions of main and interaction effects in output for HIF1α·HIF1β·PHD as shown in Figure 5.11.

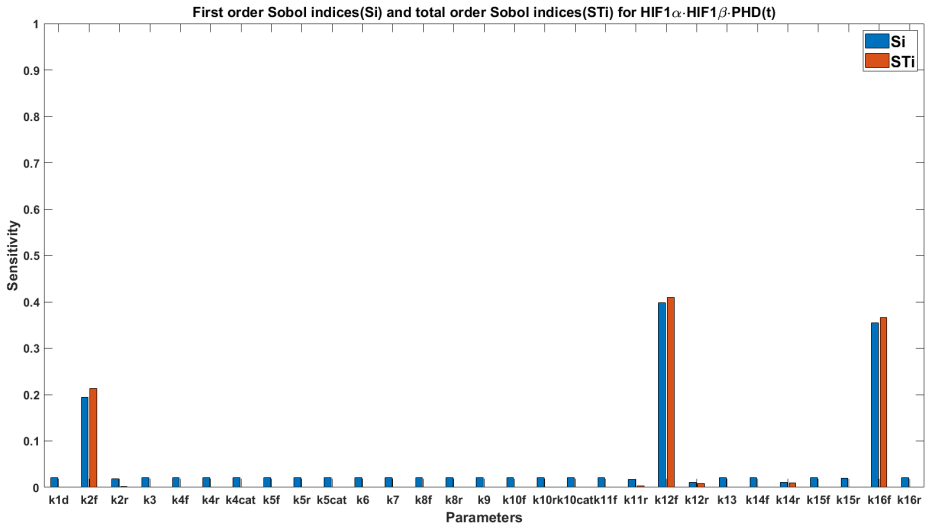

Figure 5.11. First- and Total-order of Sobol's Indices for HIF1α·HIF1β·PHD

Figure 5.12 indicates that k_{4f}, k_{14r}, k_{15r} and k_{16f} are most influential on both main and interaction effects than on HIF1α_{OH}·VHL, while k_{2f} and k_{12f} have smaller effect than k_{4f}, k_{14r}, k_{15r} and k_{16f}.

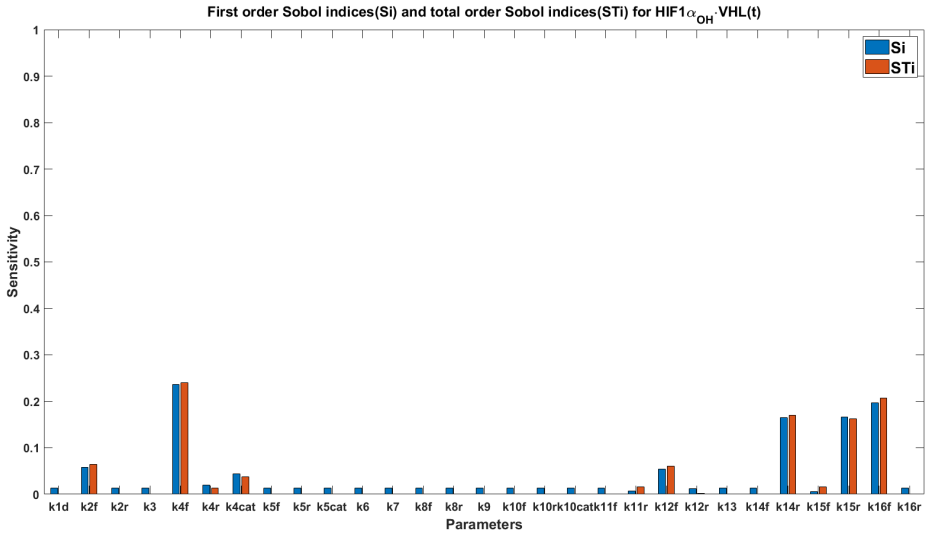

Figure 5.12. First- and Total-order of Sobol's Indices for HIF1α_{OH}·VHL

The parameters k_{14r} and k_{16f} have larger impact than k_{2f} and k_{12f} on HIF1α_{OH}·HIF1β in Figure 5.13.

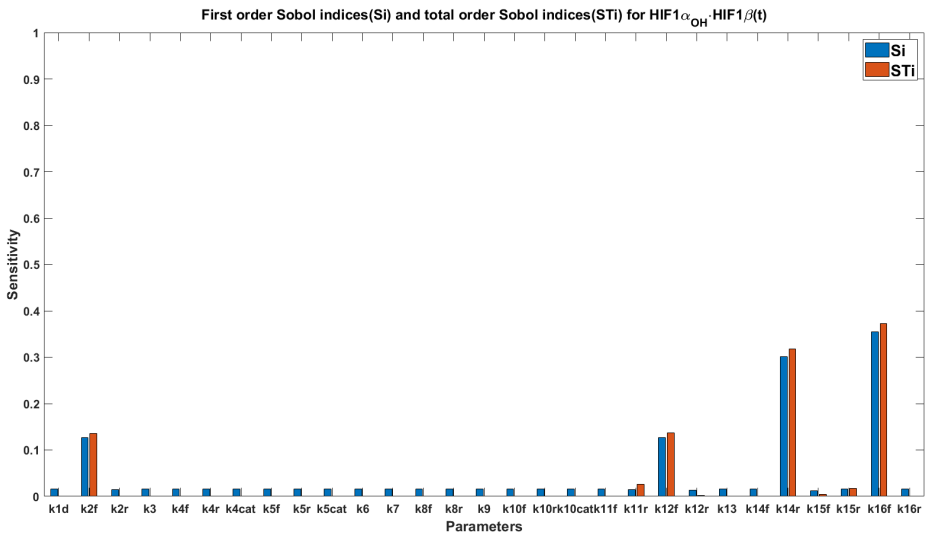

Figure 5.13. First- and Total-order of Sobol's Indices for HIF1α_{OH}·HIF1β

Figure 5.14 illustrates that k_{2f}, k_{12f} and k_{16f} are most influential on both main and interaction effects for the HIF1α·HIF1β.

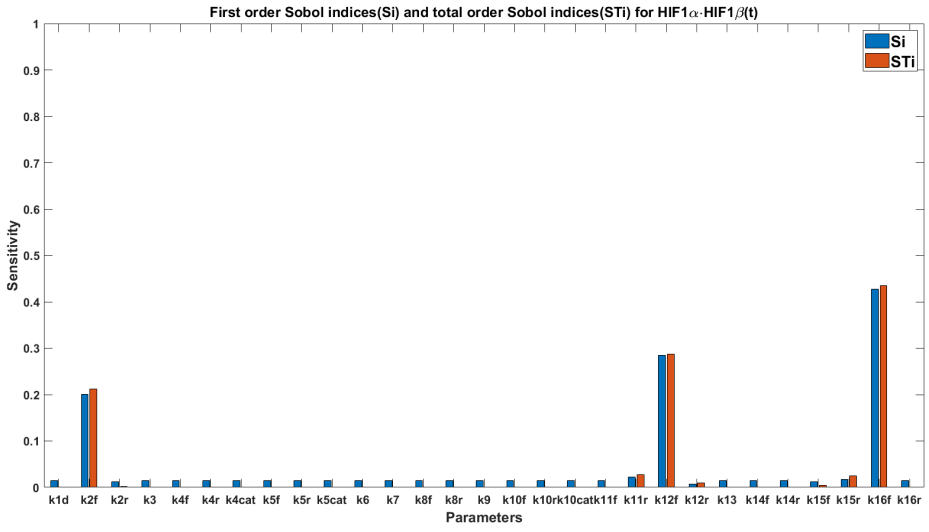

Figure 5.14. First- and Total-order of Sobol's Indices for HIF1α·HIF1β

Figure 5.15 denotes that the parameters k_{12f}, k_{14r}, k_{15f}, k_{15r} and k_{16f} both have major contribution on main and interaction effects compared to k_{2f} for HIF1α·HIF1β·HRE complex.

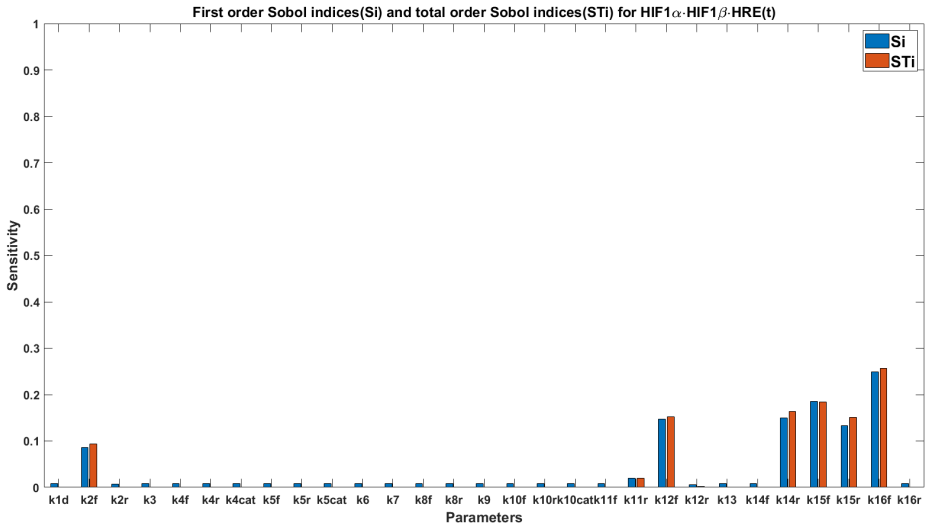

Figure 5.15. First- and Total-order of Sobol's Indices for HIF1α·HIF1β·HRE

The parameter k_{16f} has the most important main and interaction effects on both HRE and HIF1α_{OH}·HIF1β·HRE complex in Figures 5.16-5.17.

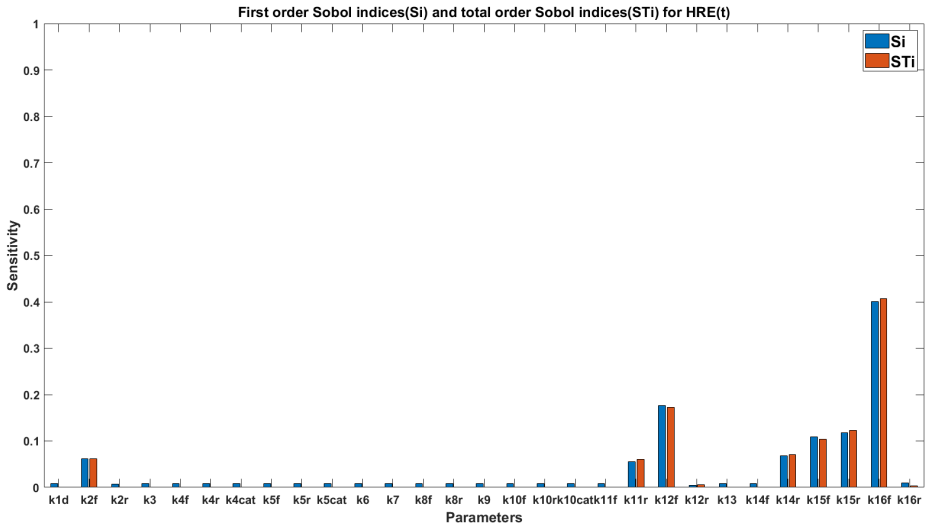

Figure 5.16. First- and Total-order of Sobol's Indices for HRE

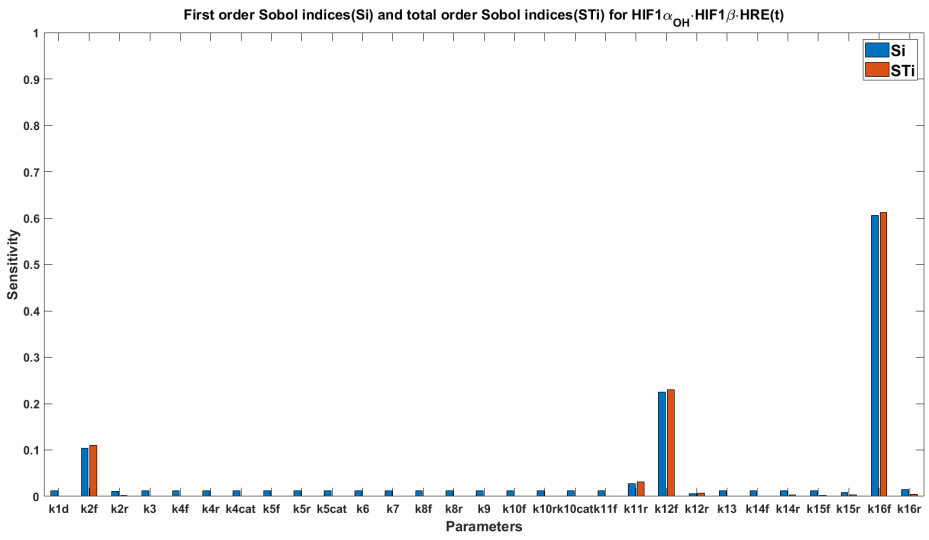

Figure 5.17. First- and Total-order of Sobol's Indices for HIF1α_{OH}·HIF1β·HRE

It can be observed from GSA results that the input parameter k_{16f} has the most critical influence on all the output variables that are involved in the genes expression; the k_{2f} and k_{12f} influence HIF hydroxylation of HIF1α and HIF1β. The sensitivity results investigate a combination of biological and mathematical experiments. Based on the parameters of variation, the found sensitive parameters, and the stability of the healthy steady state, the dominating parameters for each experiment may be recognised.

5.5 Conclusion

It has been well established that SUMOylation of protein is involved in almost all aspects of cell biology and plays a crucial role in various biological processes. In hypoxia response, it has been implied that SUMOylation of HIF1α is triggered by hypoxia stress. In recent years, experimental studies have reported that SUMOylation, as a PTM, is a critical factor of cell fate in response to extreme stress, including hypoxia stress [28, 29]. Most critically, all evidence suggests that process of SUMOylation and deSUMOylation is an essential in cellular response to hypoxia. Although much progress has been made in understanding the mechanism of SUMOylation/deSUMOylation under low oxygen tension, there is still much debate, and many unsolved questions [42].

This chapter has developed mathematical modelling and experimental validation for studying cell response to hypoxia. The author has focussed on the mechanism of SUMOylation

of the hypoxia-inducible factor 1α (HIF1α) and SENP1 level in the biological process. The hypoxia response models have been determined over the past decades. Kohn *et al* [19] proposed molecular interaction maps (MIMs) as a diagrammatic pattern for representing gene regulatory networks in a comprehensible manner. They segregated a smaller key subsystem from the whole MIMs to reduce the model's complexity. HIF1α, HIF1β, PHD, VHL, and hypoxia response element (HRE) made up the core factors. The rate constants were discovered by searching the parameter space for the HRE occupancy sharpest switch-like response as a function of the oxygen tension (5% and 10%, respectively). However, the contribution of SUMOylation in hypoxia response is never untouched concerning mathematical models. It is the first time the mechanism of SUMOylation in hypoxia response has been proposed by mathematical modelling methods. In this chapter, similar to the SUMOylation-dependent model of HS, using the law and mass action and Michaelis-Menten kinetics with the underlying signalling network inferred from experimental findings and the model parameters have been optimized by a GA-based data-driven approach. The biochemical rate reactions are converted into a system of ordinary differential equations (ODEs), which described the hypoxia process. Time-course experiments were designed and carried out in Dr Chun Guo's laboratory. Moreover, global sensitivity analysis (GSA) of Sobol's methods is employed for the impact between parameters and output variables. From the GSA results, input factor k_{2f} is most influential on hydroxylation of HIF1α; k_{12f} and k_{14r} impact on the process of activating target genes' transcription activity by HIF1α· HIF1β complex; and k_{16f} affects almost the entire SUMOylation of hypoxia process. These results illustrate that changes to the outputs can be achieved by tuning the input factors that have the most considerable impact, narrowing the 29 parameters down to a few. The preliminary results have shown that the findings are qualitatively consistent with the experimental observations by comparing normalized simulation results with our experimental data. Quantitatively the response of the predicted outcome is lower than the experimental data. This may be caused by several factors such as the mismatch of the model structure, the inadequate reaction rates, unknown biological reactions, or the GA method stuck in local minima.

Several curious conditions that have not yet been tested experimentally can be addressed by simulating the developed mathematical model. In Figure 5.3, the developed model was used to observe the consequence of the SUMO conjugation (HIF1α_s) level under 1%

oxygen tension. The SUMO conjugation level increases successively at the start of the simulation and reaches the peak value in around 6 h. After that, the level remains stable, showing that hypoxia might enhance the stability of HIF1α_s activity. In Figure 5.4, the developed model was accustomed to predict the result of the SUMO conjugation level under three different oxygen tension, 1%, 0.5% and 0.1%. The lower oxygen tension is, the higher level of SUMO conjugation, which indicates the essential correlation between oxygen tension and SUMOylation level.

SUMOylation has emerged as a critical regulator of various cellular signalling pathways [41], which is caused by cellular stressors such as hypoxia, and might be a crucial biochemical pathway for cells to survive in response to various stimuli. SUMOylation regulates several biological processes, including gene transcription, DNA replication and repair, chromosomal segregation, and protein transport via the nuclear pore [219][304]. Furthermore, SUMOylation has been associated with the molecular processes of neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's. Therefore, establishing the mathematical model of SUMOylation-development synergism of hypoxia response offers a novel perspective and possibilities for the subsequent treatment of related human diseases.

Chapter 6

Conclusions and Future Work

SUMOylation is a rapidly reversible post-translational modification process in organisms, which regulates the transcription of genes, DNA replication and repair and cellular stress response. Therefore, it has a pretty significant impact on the life activities of living organisms and is involved in the determination of cell fate upon extreme stress. It is necessary to discover the mechanisms and pathways behind SUMOylation more clearly. The journey to explore mechanisms of SUMOylation and deSUMOylation in cells has been decades. Several enzyme families are known for the process. When cells are under severe stresses, such as heat shock and hypoxia, SUMOylation is one of the mechanisms to defend against injury and protect cells at the expected activity level. In this thesis, to more accurately understand the stress-induced SUMOylation process, two mathematical models based on biological processes in which cells respond to different stresses: heat shock (HS) and hypoxia, have been established, respectively. Given the complex architecture of biological systems, mainly signalling networks, model-based approaches can help tease out the emergent network properties and identify the governing conditions. Mathematical modelling and simulation can also be used in assessing the viability of potential therapeutic strategies by predicting the response to drugs targeting these networks in related pathologies. Moreover, the integration of modelling and experimental approaches can alleviate the considerable logistic challenges associated with pure experimental methods.

6.1 HS Model

The need to understand the cells' detecting, reacting, and defending processes in response to extreme environmental and disease situations has risen dramatically in recent years. For survival, organisms need to be fit and capable of adapting to severe stressful environmental and pathophysiological conditions. The heat shock is one of the most common stressors that represent a significant obstacle to life [3–5], it can be triggered by a temperature increase of only a few degrees (°C), and the relationship among sensitivity to small changes in temperature, exposure duration and damage extent are nonlinear.

In response to heat shock, cells activate an ancient signalling pathway leading to the transient expression of heat shock proteins (HSPs), which protects the cell from the cytotoxic stress or damage due to misfolded proteins and helps it repair with heat shock response (HSR) [387]. It is now known that the core players in the HSR are HSPs, which serve as molecular chaperones assisting in the refolding of denatured proteins. While the upregulation of HSPs is through the gene expression of a specific group of transcription factors upon heat shock, known as heat shock factors (HSFs) [9]. HSF1 is regarded as a critical regulator in the HSF family. During unstressed conditions, HSF1 exists as an inactive-monomer form, while under stressful conditions, HSF1 forms an active trimer that has the ability to bind the heat shock element (HSE) in the HSP promoters region of the heat shock genes to regulate transcriptional expressions of heat shock genes to promote expression of HSPs [388].

Although the roles of the process of HSR are well documented, early signalling mechanisms in the HSR have yet to be fully understood. Interestingly, recent experimental work has suggested that SUMOylation as one PTM type is a key determinant of cell fate in response to these extreme stresses, including heat shock stress [28]. It has been implicated in the regulation of a host of cellular processes, and is essential for the health, and even the survival, of most organisms. Current evidence shows that increased SUMO-2/3 conjugation following heat shock is essential for cells' survival [36]. In addition, HS is one of the most widely characterised stresses in eukaryotes and is correlated to diverse diseases, including neurodegenerative diseases and cancers [7].

6.2 Hypoxia Model

A vast number of damaged cellular structures and denatured or misfolded proteins can result from severe stresses if organisms lack a proper stress response. Therefore, understanding how cell fate is determined when exposed to extreme stress, such as hypoxia, is crucial in biological systems. Hypoxia is present in both physiological and pathophysiological conditions [5]. Oxygen occupies a central role in the maintenance of life; however, oxygen transport by simple diffusion becomes limiting as organisms become larger and more active [155]. In order to maintain oxygen homeostasis, advanced eukaryotes have adopted unique mechanisms to enhance O_2 absorption, and distribution [23]. For this reason, mechanisms have evolved for eukaryotic cells to adapt to conditions where oxygen demand exceeds supply [156]. These mechanisms rely on modifying pre-existing proteins, translational arrest and transcriptional changes. Alterations in gene expression and cellular metabolism can be caused by hypoxia. To investigate the detailed mechanisms of hypoxia, Kohn *et al.* demonstrated the molecular interaction maps (MIMs) of the hypoxia response pathway [19][21]. The critical factor in this network, the hypoxia-inducible factor (HIF), is the master regulator of oxygen-sensitive gene expression. HIF is a heterodimeric transcription factor consisting of one of the three members: HIF1α, HIF2α and HIF3α; and a common constitutive ARNT subunit which is also known as HIF1β. The system also includes an enzyme family: prolyl hydroxylases (PHDs), which directly sense the oxygen level and hydroxylate HIF1α by covalently modifying the HIFα subunits [23, 24]. The hydroxylated HIFα is subsequently subjected to degradation by VHL action. After that, the complexes of HIF1α and HIF1β are located in hypoxia responsive element (HRE) for genes expression [161, 162].

Post-translational modifications as crucial factors in cellular signalling that enable to regulate of protein functions [42]. SUMOylation as one PTM type is a key determinant of cell fate in response to these extreme stresses, including hypoxia stress [28]. SUMOylation is a dynamic PTM process where small ubiquitin-like modifier (SUMO) proteins are covalently attached to target proteins in cells to modify their functions in all eukaryotes. It has been implicated in regulating a host of cellular processes and is essential for most organisms' health and even the survival of most organisms, [30]. Studies have shown that SUMO targets are involved in various aspects of cell biology while controlling nuclear transport

mechanisms. SUMOylation, one of the most common reversible protein modifications, is involved in cellular processes such as signalling, transcription, recombination, chromosome segregation and DNA repair [42]. Furthermore, recent research shows that stabilising and activating the transcription factor HIF1α may have neuroprotective benefits while also triggering hypoxic signalling pathways [48]. As a result, activating the HIF1α signalling pathway and raising the expression of its protective target genes might be a sound neuroprotective strategy for the treatment or prevention of neurodegenerative diseases [196].
To study the complicated dynamic behaviour of hypoxia biological systems, mathematical models and data-driven approaches such as genetic algorithm (GA) and global sensitivity analysis (GSA) are emerging as promising tools for such purpose [389].
Overall, although the mathematical models of HS and hypoxia have been established for predicting outcomes that are consistent with experimental results, there are some limitations in the HS model and hypoxia model. Firstly, for the HS model, there are very few time courses data available in the literature for the SUMOylation of HSF1 trimer in response to HS. In this thesis, only two sets of data from the literature [9][36], and three sets of data from our experiments were considered. For the hypoxia model, the time courses data for the SUMOylation of HIF1α and SENP1 in response to hypoxia are also very limited availability in the literature. In this thesis, SUMOylation of HIF1α data from the literature [25] and SENP1 data from the [43], and three sets of data of SENP1 for our experiments are given. The global SUMOylation of HIF1α is hard to detect through the experiment; thus, only SUMOylation of SENP1 is considered in the experimental validation. However, the SUMOylation of HIF1α tendency cab is obtained from the simulation predicted results. This limits the benefit of using data-driven methods, which need to be trained with a large amount of data to perform well. In this regard, further experimental study about the role of SUMO proteins in response to heat shock is needed. Secondly, because the experimental data were obtained from ImageJ and Image Studio Lite, the model output had to be normalised to the maximal response. Hence, a direct comparison of the simulated concentrations is not possible. In the future, a new experimental design may be needed. Thirdly, for the HS model, there were some simplifications for the model development, including the phosphorylation of HSF1 trimer was ignored; for the hypoxia model, the degradation of PHD and VHL was ignored, and only HIF1α degradation was considered in

this model. Fourthly, the cases where SUMO is polymerised into polySUMO chains were not considered. In future studies, these aspects will be considered, included and examined.

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